

**A POSSIBLE ROLE FOR INTRINSIC
CALCIUM BUFFERS IN THE LONG-TERM
ADAPTATION OF CRAYFISH NEURONS**

by

John Christopher McMahon, B.Sc., Hons.

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**Brock University
St. Catharines, Ontario**

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ABSTRACT:

Increasing the impulse activity of neurons *in vivo* over 3 or more days causes a reduction in transmitter release that persists for days or weeks (eg. Mercier and Atwood, 1989). This effect is usually accompanied by decreased synaptic fatigue. These two changes involve presynaptic mechanisms and indicate "long-term adaptation" (LTA) of nerve terminals. Previous experiments have shown that LTA requires extracellular calcium and protein synthesis (eg. Hong and Lnenicka, Soc. Neurosci. Abstr. 17:1322) and appears to involve communication between the cell body and the nerve terminals. The present study examines the possibility that the reduction in transmitter release is caused by an increase in the calcium buffering ability within the nerve terminals. It examines the responses of adapted and control nerve terminals to exogenously applied calcium buffer, BAPTA-AM, which decreases transmitter release (Robitaille and Charlton, 1992). If LTA increases intrinsic Ca^{2+} -buffering, the membrane permeant form of BAPTA should have less effect on adapted nerve terminals than on controls.

Experiments are performed on the phasic abdominal extensor motor neurons of the crayfish, *Procambarus clarkii*. BAPTA-AM decreases excitatory postsynaptic potentials (EPSP's) of the phasic extensor muscles in a dose-dependent manner between 5 and 50 μM . LTA is elicited by *in vivo* stimulation at 2.5 Hz for 2-4 h per day over 3 days, which reduces EPSP's by over 50%. Experiments indicate that BAPTA-AM produces no significant change in EPSP reduction in adapted neurons when compared to controls. These results do not support the hypothesis that increased daily activity alters rapid intrinsic calcium buffers, that are able to reduce transmitter output in the same manner as BAPTA.

INTRODUCTION:

Nerve cells can be classified into distinct physiological types, which appear to be adapted to meet different functional needs (Atwood *et al*, 1991). Impulse activity plays a major role in the physiological properties of nerve cells and probably directs the formation of such physiological types. In general, high impulse activity adapts the neuron so as to maintain transmitter release over extended periods of time (eg. Lnenicka and Atwood, 1985 a,b; Hinz and Wernig, 1988; Atwood *et al*, 1991). Such physiological changes are accompanied by morphological and/or biochemical alterations in the mitochondria (Lnenicka *et al*, 1986; Wong-Riley, 1989) and morphological changes in the nerve terminals (Lnenicka *et al*, 1986; Pawson and Grinnel, 1990).

This thesis is concerned with the mechanisms which underlie the physiological changes that neurons exhibit in response to increased electrical activity. It examines the possibility that activity greatly alters the neuron's intrinsic ability to buffer calcium ions. Calcium ions are likely to be involved in such activity-dependent processes for at least three reasons. First, electrical activity in neurons usually triggers an influx of calcium ions into the cytoplasm, from the extracellular milieu (eg. Llinás and Nicholson, 1975; Llinás *et al*, 1981; Miledi and Parker, 1981; Miledi *et al*, 1982; Stuenkel, 1990). Second, calcium influx plays a crucial role in triggering transmitter release. It also determines the number of quanta released (quanta in this case meaning

transmitter released from individual vesicals) (eg. Hutter and Kostial, 1954; Katz and Miledi, 1964; Richard and Sercombe, 1970; Miledi, 1973; Llinás and Nicholson, 1975). Third, intracellular calcium regulates other cellular processes, such as protein synthesis, phosphorylation and protease activity (eg. Nestler and Greengard, 1982; Roach, *et al*, 1987; Mills and Kater, 1990; Silver *et al*, 1990; Weiss, *et al*, 1990; Sheng *et al*, 1991). Protein synthesis and phosphorylation have been implicated in establishing long-lasting changes in transmitter release in response to activity (Nguyen and Atwood, 1990).

The question of whether cellular activity regulates calcium buffering, however, is of general significance because calcium is used as an intracellular signal in virtually all cell types.

Tonic and Phasic Properties.

Crustacean motoneurons can be classified as either phasic or tonic. Tonic motoneurons innervate tonic (slow) muscle and maintain low level activity at most times. Contraction is slow and has great fatigue resistance (Atwood, 1973). Phasic or fast motoneurons innervate fast muscle, such as the deep abdominal flexor and extensor muscles used in escape swimming. These muscular responses are rapid, but fatigue quickly (Atwood, 1973). The reasons for the response differences can be seen when excitatory post synaptic potentials (EPSP's) are measured from the muscle. When repetitively activating a phasic neuron, the EPSP's are initially very large due to high transmitter release. The EPSP's rapidly decrease in amplitude due to synaptic

fatigue. When tonic neurons are activated at a high frequency (>5 Hz), EPSP's are initially small, but increase due to synaptic facilitation. Synaptic fatigue develops slowly in tonic neurons (Atwood, 1976). In addition there are morphological differences between tonic and phasic nerve terminals. The tonic terminals are more varicose and their mitochondria take up more volume (Lnenicka *et al*, 1986).

In some vertebrates, tonic muscle fibres are similar to those of invertebrates, being multiterminally innervated, frequently non-spiking and can sustain tension for prolonged periods (Hess, 1970). Consequently what is discovered in the simpler invertebrate system may be transferable to the more complex vertebrate nervous system, and of course *vica versa*.

Long Term Adaptation.

Previous studies have shown that phasic and tonic neuron types can be modified. That is, phasic neurons can be made to act and look more like tonic neurons by increasing their electrical activity. This type of synaptic plasticity, referred to as Long Term Adaptation (LTA), has been investigated extensively in crayfish and lobsters (Bradacs *et al*, 1990, Lnenicka and Atwood, 1985 a,b, Lnenicka *et al*, 1986, Mercier and Atwood, 1989). The physiological properties of crayfish phasic closer motoneurons can be shifted towards even more phasic characteristics if they are prevented from firing (Pahapill *et al*, 1985). This appears to be a reversal of LTA and has been studied in crayfish as well as in mammals (eg. Gallego *et al*, 1979; Robbins and Fischbach, 1971).

If the fast closer muscle in the crayfish claw is stimulated at 5 Hz, 2h/d for 3-4 days the following changes are observed: a) less transmitter release during initial stimulation; b) transmitter release is sustained over prolonged stimulation; c) mitochondria in the nerve terminals increase in size; d) mitochondria are in greater proximity to the synapses. These changes are characteristic of LTA (Lnenicka and Atwood, 1985 a,b, Lnenicka *et al*, 1986).

There is evidence that these synaptic changes involve protein synthesis. First, Nguyen and Atwood (1990) found that *in vivo* injection of cycloheximide (CHX), a potent inhibitor of protein synthesis, during a period 2-6 hours prior to stimulation prevented changes in transmitter release. Injection of CHX outside of this critical "window" did not effect LTA. Furthermore, if the nerve terminal is physically isolated from the cell body, which is the main site of protein synthesis, LTA is not observed (Lnenicka and Atwood, 1985 a). Thus, some signal from the cell body appears to be necessary for LTA. If the cell body is stimulated antidromically (from the nerve terminal toward the cell body), or if the spread of impulses to the nerve terminals is blocked using tetrodotoxin (TTX), a sodium channel blocker, LTA still occurs (Lnenicka and Atwood, 1989). Thus, depolarization of the cell body and/or dendrites is sufficient to elicit LTA. Together these results suggest that synaptic changes are mediated by proteins synthesized in the cell body and transported down the axon to the synaptic terminals.

Nguyen and Atwood, (1992) found that if protein synthesis is blocked after conditioning the neuron, LTA still occurs. Under these conditions

however, fatigue resistance is lost at a more rapid rate than is the recovery of transmitter output. This indicates that more than one signal may be sent to the terminals. If so, it could be speculated that one signal would be short lived and would reduce the amount of transmitter released per stimulus. The other signal would be either longer lived or continuous in nature, and would enhance the fatigue resistance of the terminals.

Similarities to LTA in other organisms.

In the frog cutaneous pectoris muscles, Hinz and Wernig (1988) found that increasing electrical activity causes physiological changes very similar to those of LTA. If stimulated for several hours per day for 5-8 days the EPSP's and quantal measurements are reduced in a manner similar to that observed in crayfish.

More examples have been found of effects similar to 'reverse LTA' in other organisms. Work on the cat (Gallego *et al*, 1979) has shown that if a tonic motoneuron is forced to rest by the chronic application of TTX, the neuron is changed from having tonic characteristics to having phasic characteristics. The muscles display large initial EPSP's which diminish rapidly upon repeated stimulation. Similar effects are also observed in other organisms. In guinea-pig sympathetic neurons, if the nerve terminals are blocked from receiving signals with TTX, EPSP's increase in amplitude (Gallego and Geijo, 1987). Sensory deprivation causes increased transmitter release and increased synaptic fatigue in the developing sensory system in

locusts (Bloom and Atwood, 1980). These examples indicate that LTA may be a widespread phenomenon, reflecting a general property of neurons.

Seasonal Variability.

Lnenicka and Zhao (1991) examined the physiology and morphology of crayfish motor nerve terminals at various times of the year. These authors found a distinct difference in the properties of the terminals depending on the season. They assessed initial excitatory post synaptic potential (EPSP) amplitudes, synaptic fatigue, and the frequency of synaptic varicosities along the nerve terminals of an identified phasic motoneuron from animals collected over a period of 5 years. In winter animals, stimulation produced large initial EPSP's which fatigued easily. In summer animals, there were smaller initial EPSP's that were much more resistant to fatigue. Also summer animals have more synaptic varicosities, as is also found in animals which have been artificially stimulated to produce LTA.

Frog motor nerve terminals (Pawson and Grinnell, 1989) also show seasonality. Transmitter release is larger in the winter months (Dec-Feb) than in spring and summer months (Mar-Aug). This conclusion is based on data collected over a 2.5 yr period and used 298 identified endplates from 40 sartorius muscles.

Role of Calcium.

Calcium is used in organic systems as a molecular bridge. Due to its size, the outer shell of electrons is fairly flexible, which can deform and join two compounds together. These calcium-mediated cross linkages have the benefit of being reversible, while disulphide bonds and sugar-peptide linkages are harder to break. This means that calcium linkages are very quick to adapt to the changing conditions within a cell (Bianchi, 1968).

Intracellular calcium is a very important regulator of various functions. It is involved in phosphorylation activation, transmitter release, mitosis, stimulation of proteolytic enzymes that contribute to cell death and in the interaction of actin and myosin in muscular contractions (eg. Katz and Miledi, 1964; Miledi, 1973; Llinás and Nicholson, 1975; Nestler and Greengard, 1982; Roach, *et al*, 1987; Willians *et al*, 1987; Mills and Kater, 1990; Silver *et al*, 1990; Weiss, *et al*, 1990; Westerblad and Allen, 1991; Sheng *et al*, 1991). Generally there is a 10,000 fold difference between extra-cellular calcium and intra-cellular calcium concentrations. In animals extra-cellular calcium is generally 2-17 mM, and intracellular calcium is in the range of tens to hundreds of nM, depending on whether the cell is active or at rest (see below). Vertebrates seem to be on the low end of the extra-cellular concentration scale (eg humans at approximately 5 mM) and invertebrates seem to generally be at the upper end (eg. Lobster at approximately 15 mM) (Eckert *et al*, 1988).

Methods For Determining Intracellular Calcium.

Conclusions about calcium's role in intracellular processes all depend on the ability to measure intracellular free (i.e. unbound) calcium and to demonstrate changes in free calcium levels during normal physiological activity. Several such methods have been developed over the years. One technique, cell impalement with calcium sensitive microelectrodes, is not the best since it has at least three major disadvantages: a) calibration problems (Otto *et al*, 1985); b) measurement at only one point inside the cell; c) intrusiveness (since any damage to the cell causes calcium entry due to the high concentration gradient from the outside to the inside).

Other techniques, which employ fluorescent calcium probes, are non-intrusive. A problem with these techniques is excitation at wavelengths that also cause autofluorescence. Digital enhancement, using analog to digital conversion, rectifies some of the problems of noise caused by autofluorescence by comparing digital images of cells that have the calcium probe to images of cells that do not. Using a computer the two images are overlaid and autofluorescence is removed. A problem with digital imaging is attenuation of the fluorescence from areas that are out of focus. Confocal imaging makes use of an illumination pinhole and a detector pinhole having a common focus. This means that a defocused image disappears instead of blurring as in standard microscopes. This method alleviates the problem mentioned above and produces a higher resolution image that reflects free calcium levels at various locations within the cell. Both digital enhancement and ratio imaging

can be used in conjunction with this technique to produce a more accurate picture of where and when inside a cell the concentration of calcium is changing (eg. Hernández-Cruz *et al*, 1990). Some examples of the uses of such probes determining intracellular calcium are presented below.

Using the fluorescent probe n-aequorin-J, Llinás *et al*, (1992) mapped microdomains of calcium entry into the presynaptic terminal of the squid giant axon. They found that the concentration of calcium was not uniform throughout the terminal after stimulation, but was raised to high levels (200 to 300 μM) in discrete areas. These microdomains remained spatially fixed throughout repetitive stimulations of the nerve. These microdomains are thought to occur in the same areas as active zones the sites where exocytosis is most likely to occur (Robitaille *et al*, 1990).

Using the fluorescent probe Fura-2, resting calcium levels were determined by Allen *et al*, (1992) in corpus allatum cells from *Manduca sexta* larvae. The free calcium concentration was found to range between 20 nM and 110 nM depending on the life cycle of the larvae.

Westerblad and Allen (1991) used Fura-2 to estimate calcium concentrations in mammalian muscle fibres. The calcium level was found to be 30 ± 3 nM at rest but rose to 967 ± 89 nM when the muscle was stimulated at 100 Hz. The authors were looking for the cause of fatigue in muscle and were able to determine that even with high calcium levels present, muscle tension was low. They concluded that fatigue produced by repeated tetani involves a combination of reduced maximum tension-generating capacity,

reduced myofibril Ca^{++} sensitivity, and reduced Ca^{++} release from the sarcoplasmic reticulum. In Purkinji neurons, Hockberger *et al* (1989) found (using Fura-2) a resting Ca^{2+} concentration of 120 nM in the dendrites and 80 nM in the somata of cells. When glutamate was added $[\text{Ca}^{2+}]_i$ rose to 200 nM in the soma and 500-900 nM in the dendrites, and the addition of 170 mM KCl caused $[\text{Ca}^{2+}]_i$ to rise to 630 nM. Thus, activation can increase free $[\text{Ca}^{2+}]_i$ between one and two orders of magnitude.

Ca^{2+} Chelators.

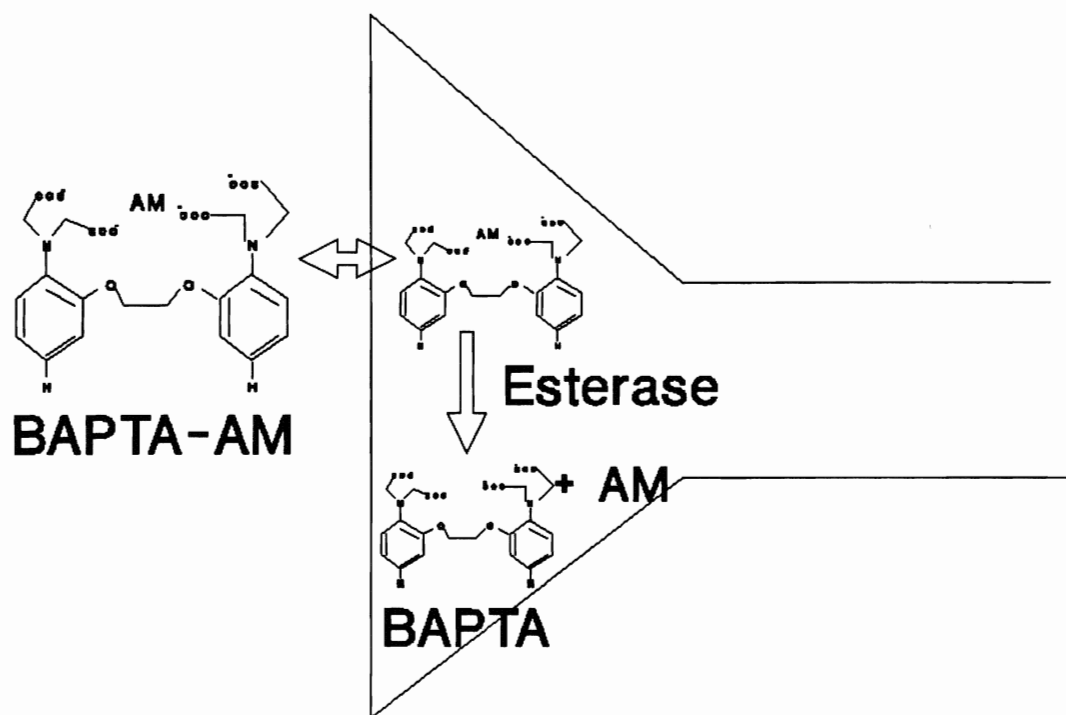
Calcium chelators which bind free calcium ions, are used experimentally to alter $[\text{Ca}^{2+}]_i$. The higher the chelator's selectivity for calcium over other ions, the more effective it is for examining Ca^{2+} -dependent processes within a cell. Chelators can be made with a variety of secondary properties as well. They can be made highly fluorescent (as are Flou-3, Fura-2, Indo-2, etc), and they can be made membrane permeant by adding an ester linkage (eg. Acetoxy Methylester). These two secondary properties make it easier to visualize the presence of calcium within membrane bound structures. There are a variety of chelators available. Three are mentioned above; another is EGTA (ethylene glycol bis (β -aminoethyl ether) $\text{N},\text{N},\text{N}',\text{N}'$ -tetraacetic acid). EGTA has a high selectivity for calcium over Mg^{2+} and H^+ but is affected by changes in pH and is slower at taking up and releasing Ca^{2+} when compared to the chelator chosen for this study, BAPTA. The structure of BAPTA is shown in figure 1.

Intrinsic Ca^{2+} Binding Proteins.

Calcium binding proteins within a cell are very important for the healthy continuance of any organism. These proteins can act as buffers regulating the concentration of calcium and keeping it at tolerable levels. If these proteins are not present or are overwhelmed by calcium, the cell can "self destruct". This occurs due to proteolytic enzyme function being triggered by the presence of excessive ion levels. Thus, calcium must be kept at a very low level within the cell compared to the exterior milieu, where free calcium levels are at least 10,000 times higher. This suggests that the cell may detect disruption and extent of damage to its external protection (membrane or wall) by the amount of Ca^{2+} present within the cell.

As well as triggering the self destruction of the cell, calcium regulates secretion, and through calmodulin, activates adenylate cyclase (Alberts *et al*, 1983). Adenylate cyclase in turn regulates cAMP production, and is a transmembrane protein (Alberts *et al*, 1983). Calmodulin activated adenylate cyclase has been found in neural tissue (Brostrom *et al*, 1975; Evain *et al*, 1979; Westcott *et al*, 1979), the adrenal medulla (Valverde *et al*, 1979) and pancreatic islets (Hewlett & Wolff, 1976). Also bacterial adenylate cyclase is affected by calmodulin (Wolff *et al*, 1980). As well as activating such systems, calcium binding proteins isolated from bovine brain has been shown to inhibit protein kinase C (McDonald and Walsh, 1985). Protein kinase C has been shown to: i) recruit hidden calcium channels (Strong *et al*, 1987); ii) augment evoked transmitter release (Shapira *et al*, 1987).

Figure 1: Structure of BAPTA-AM. The cell permeant form of BAPTA (BAPTA-AM) is shown entering the nerve terminal. After entering the terminal, intrinsic esterases cleave the acetoxy methyl ester, leaving the impermeant salt form of BAPTA which can then bind Ca^{2+} ($K_m \approx 10^{-7} \text{ M}$)



Mediation of Transmitter Release.

Transmitter release is triggered by a transient stimulus-induced rise in intracellular Ca^{2+} entering the nerve terminal through channels located in the presynaptic membrane (Katz, 1969; Augustine *et al.*, 1987).

There is evidence that calcium is necessary and sufficient to elicit quantal release of neuro-transmitter. Early work showed that in a calcium deficient environment, localized application of calcium from a micropipet causes a large increase in the quantal component of the end-plate potential within less than 1s (Katz and Miledi, 1965). Later (Miledi, 1973) showed that when calcium was injected into the squid giant axon, transmitter release occurred, but the injection of Mn^{2+} or Mg^{2+} did not elicit release. When Ca^{2+} was replaced in the external environment with Mn^{2+} depolarization of the nerve terminals failed to elicit transmitter release. Thus, depolarization appears to trigger influx of Ca^{2+} into the terminals which, in turn, triggers release.

Recent work re-examining the hypothesis that calcium is sufficient to cause release of substances from the nervous system has been done. Work by Nordmann and Stuenkel, (1991) has demonstrated calcium-independent neurosecretion in isolated rat neurohypophysial nerve endings. They found that elevation of secretion is caused by a rise in sodium concentration in a dose-dependent manner and is not related to changes in Ca^{2+} concentration. This does not show that calcium is not involved in transmitter release, but rather that factors other than calcium may also be involved.

Other studies add to the controversy over the importance of calcium in transmitter release. Parnas (1985) hypothesized that a voltage component as well as a Ca^{2+} component are both involved in triggering release. This hypothesis has been debated mainly between Parnas and Zucker. Zucker and Haydon (1988) examined the effects of voltage and Ca^{2+} on transmitter release from cultured neuronal cell bodies. This preparation allows experimental manipulation of membrane voltage (through voltage clamping) near the synapses. They also manipulated intracellular Ca^{2+} levels by loading the cells with "caged-Ca" molecules that release Ca^{2+} when illuminated at a specific light wavelength. Such "flash-illumination" elicited transmitter release, but the amount of such release was not affected by transmembrane voltage. Thus, the signal for release in these neurons appears to be a rise in intracellular free Ca^{2+} and not a change in voltage. Hoenner *et al*, (1989) applied similar methods with a higher release crayfish nerve and found a correlation between voltage and transmitter release. They hypothesized that depolarizing the membrane causes a molecule to change conformation. This conformational change makes the molecule sensitive to Ca^{2+} , when the terminal is repolarized the molecule becomes insensitive to Ca^{2+} and transmitter release is terminated. In reply Mulkey and Zucker (1991) used the same preparation, and found that transmitter release is unaffected by presynaptic action potentials.

The location of the Ca^{2+} channels are important in that Ca^{2+} is thought to diffuse slowly in the cytoplasm and is rapidly sequestered (Nasi and Tillotson, 1985; Donahue and Abercrombie, 1988). The position of calcium

channels at the frog neuromuscular junction (nmj) relative to the position of transmitter release was examined by Robitaille, *et al* (1990). Fluorescently tagged ω -conotoxin GVIA, which binds irreversibly to Ca^{2+} channels, was used to identify the location of the channels. Fluorescently tagged α -bungarotoxin was used to label acetylcholine receptors, which are situated on the muscle membrane directly opposite the active zones of transmitter release. The authors found that the Ca^{2+} channels lined up almost perfectly with the acetylcholine receptors. This indicates that groups of Ca^{2+} channels are localized exclusively at the active zones of the frog NMJ (Robitaille, *et al*, 1990). The observation that the calcium channels are so close to the site of transmitter release supports the idea that calcium entry and transmitter release are strongly linked. It also makes it possible to propose the idea that the drop in transmitter observed due to LTA could be linked to some aspect of calcium entry. The close proximity of the calcium channels to the sites of release could also account for the very short latency between the influx of calcium and release of transmitter (approx. 200 μs).

Possible Mechanisms of LTA

The two components of LTA, decreased transmitter output and increased fatigue resistance, have been shown to be separable from each other (Nguyen and Atwood, 1992, Bradacs *et al*, 1992). If the changes can be separated from each other it may be that the mechanisms responsible could be different. The increase in fatigue resistance could be related to the increased

mitochondrial volume reported by Lnenicka *et al* (1986). This increase in volume could increase the amount of ATP present within the nerve terminals which may lead to an increased ability of the terminal to maintain ionic gradients during sustained activity.

There are a number of mechanisms that could be involved in the reduction of transmitter release: a) increased buffering of intracellular calcium, b) reduced Ca^{2+} entry, c) decreased response of the transmitter release mechanisms to intracellular Ca^{2+} , and d) a decrease in the excitability of the nerve terminals.

This thesis examines the first of the above mechanisms in detail. This possibility is suggested by observations that applying rapid, mobile calcium buffers intracellularly, can reduce transmitter release (eg. Robitaille *et al*, 1989; Adler *et al*, 1991). The central hypothesis is that if the free Ca^{2+} buffering capacity within the nerve terminals changes, then the transmitter output of the terminals would also change.

In theory, a reduction in Ca^{2+} entry can be accomplished in a number of ways. The calcium channels could be blocked, or they could be degraded. Alternatively, the calcium influx could be reduced by changes in membrane voltage due to increased potassium currents, such as those associated with Ca^{2+} -activated K^+ channels. The ability of Ca^{2+} -activated potassium ($g_{\text{K}(\text{Ca})}$) channels to modulate synaptic transmission has been demonstrated in the frog neuromuscular junction by Robitaille and Charlton (1992). Charybdotoxin (CTX) a blocker of $g_{\text{K}(\text{Ca})}$, induced a twofold increase in transmitter release;

apamin, which blocks different K^+ channels did not have an effect. CTX was ineffective after the addition of BAPTA-AM, which presumably prevents activation of the $g_{K(Ca)}$ channels by chelating intracellular Ca^{2+} ions. Interestingly the chelator alone caused an initial increase in transmitter release followed by a decrease in transmitter release. The increase did not occur in the presence of CTX or Ba^{2+} . Using a calcium sensitive fluorescent probe (FLUO-3) it was found that Ca^{2+} entry increased after blockage of $g_{K(Ca)}$ channels. These results indicate that $g_{K(Ca)}$ channels normally limit transmitter release by narrowing the action potential at the nerve terminal. This will shorten the time for calcium entry (Llinás *et al*, 1981, 1982; Augustine, 1990). Robitaille and Charlton, (1992) also state that in order to affect the presynaptic action potentials, the $g_{K(Ca)}$ channels must be located close to the Ca^{2+} channels.

The third hypothesis can be related to work done by Man-Son-Hing *et al*, (1989), though they never examined LTA. They found that FMRFamide reduces the post synaptic current (thus transmitter release), when applied to cultured *Helisoma* neurons. Even with calcium present within the nerve terminal (due to photo release of 'caged calcium') post synaptic currents were still inhibited. This means that at some point after calcium entry, transmitter output can be decreased.

The fourth hypothesis is suggested by work in which a "macropatch" electrode is used to directly depolarize the nerve terminals. When synaptic currents are measured, a difference in excitability can be observed. Phasic

lobster abdominal extensor nerve terminals, have a plateau stimulus intensity above which release saturates and below which release is not evoked (Dudel *et al*, 1984); TTX is able to eliminate this threshold. Tonic opener excitor terminals on the other hand seem to be inexcitable, with a gradual increase in stimulus intensity and an insensitivity to TTX (Dudel, 1983). With this in mind work should be done which examines the excitability of nerve terminals before and after LTA is evoked.

Hypothesis

This thesis examines only one of the possible causes of Long Term Adaptation (LTA) in the crayfish *Procambarus clarkii*. The central hypothesis is that LTA involves an increase in intrinsic calcium buffering within the synaptic terminals. This possibility is examined using a commercially available, membrane permeant Ca^{2+} chelator (BAPTA-AM). Crayfish are used as the experimental animal since LTA is well documented in them and since they are inexpensive and easy to maintain.

BAPTA was selected because it is an effective calcium buffer at low concentrations (Tsien, 1980). Ca^{2+} ions bind to BAPTA molecules in a 1 to 1 manner. Since magnesium is bound less than calcium by 5.2 log units, intracellular magnesium ions will not influence BAPTA's effect. Calcium affinity to BAPTA (K_m) is approximately 10^{-7} M, which is about the resting intracellular calcium level of many cell types (Tsien, 1980). In addition,

BAPTA binds Ca^{++} rapidly enough to lower transmitter release (Robitaille, 1991).

Some basic assumptions are that intrinsic calcium buffers have a K_m similar to that of BAPTA, are as mobile as BAPTA and are also as rapid at binding calcium. Assuming that a intrinsic calcium buffer had all three of these characteristics, then intrinsic calcium buffers would be able to lower transmitter output in much the same manner as BAPTA has been shown to do. If activity increases the concentration of such calcium buffers, then when BAPTA is added, the effect should not be as great. In other words if a terminal is at a high buffering level, then adding extrinsic buffer to it should not produce the same effect as adding the same amount of extrinsic buffer to a terminal that is not at as high a internal concentration of buffer. Thus, a comparison of the effect of BAPTA on transmitter release between conditioned and control sides of an animal can be used as an indication of whether activity alters such buffering capacity.

Animals are conditioned on one side to produce LTA in the phasic extensor motor neurons. The conditioned and control sides are then tested to see if the conditioning is effective and to determine whether or not adapted neurons respond differently to BAPTA than do control neurons. The prediction is that if activity increases rapid calcium buffering, BAPTA should substantially reduce EPSP's on the control side, but it should have a proportionately smaller effect on the conditioned side.

MATERIALS AND METHODS:

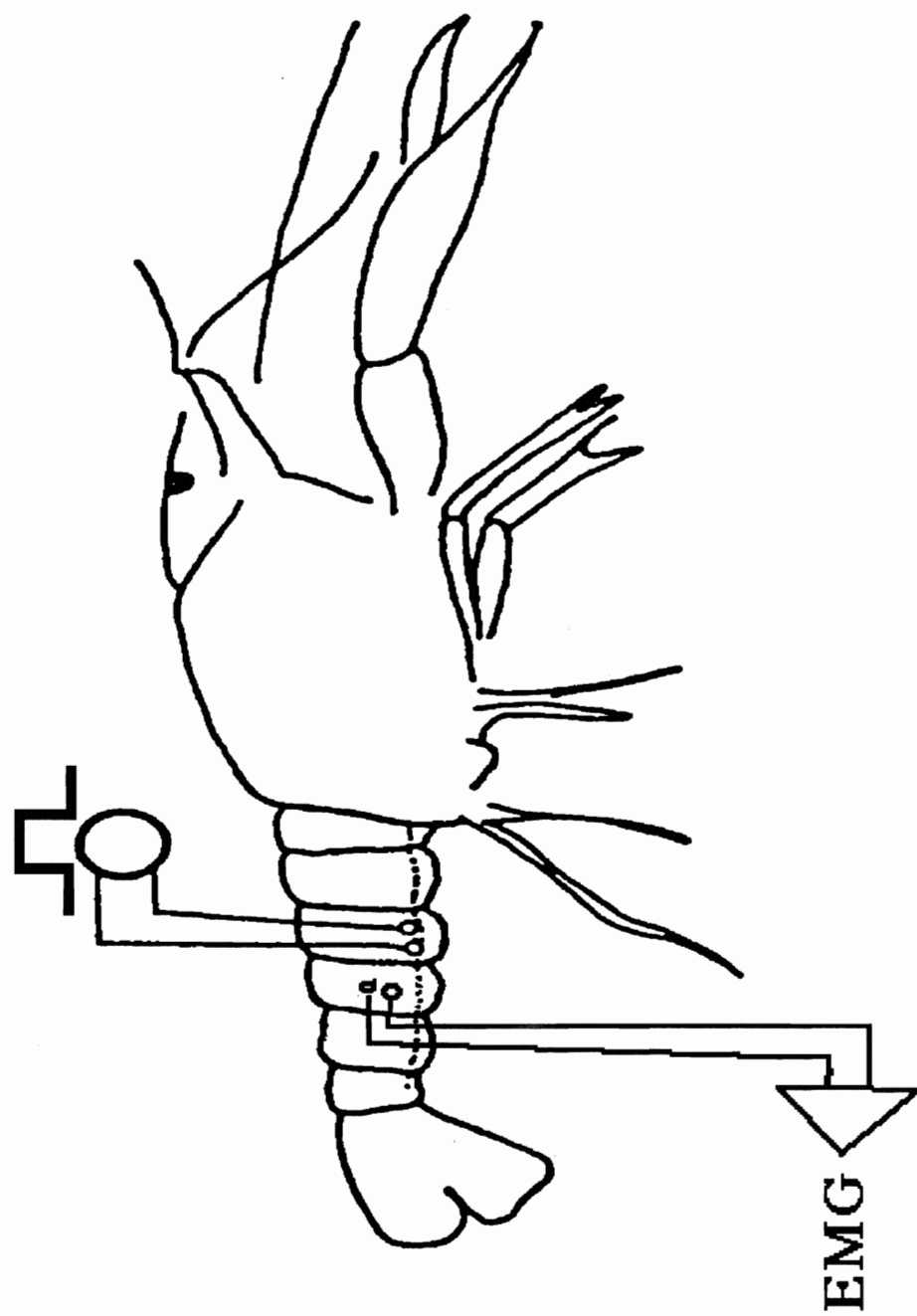
The study animal was the crayfish *Procambarus clarkii* obtained from the Atchafalaya Biological Supply Company Inc. based in Louisiana, U.S.A.. The selected animals were between moults, since the loss of the exoskeleton dislodges the conditioning electrodes. Thus, only animals with reasonably solid carapaces were used. The animals were generally 2-3 cm in length.

Conditioning

The animals were stimulated using the protocol outlined below. The stimulators used included Grass models S48 & S88F, an E&M Physiograph SI-10 stimulation module and W-P Instruments models 1830 & 1831 pulse generators. The oscilloscopes used to monitor the muscle potentials, cell resting potentials and EPSP's were Hameg 20 MHz Storage Scope HM205-3 and Tektronix models 547 & 564 oscilloscopes. Grass P15 AC preamplifiers were used for electromyographic recording during the stimulation of neurons *in vivo*.

Two holes in each of segments 3 and 4 were drilled (Figure 2) using a small hand drill (DREMEL Moto-Tool model 260 series 55-3) kindly provided by Dr. Arthur Houston. A very fine stainless steel wire (0.2 mm diameter) was then inserted into each hole with care taken not to damage the muscle underlying the carapace. These wires were then glued onto the animal using one of two protocols. The first method involved securing the wires using 5 minute epoxy (Lepag ) which was covered with CrazyGlue® and left to bond

Figure 2: Location of electrodes during the conditioning of a crayfish nerve. Stimulating electrodes are placed along the dotted line in segment three. Recording electrodes for monitoring EMG activity are placed in segment four in such a way as to bracket the L1 muscle.



for a period of 2 hours. The second method involved using 3M Vetbond™ (tissue adhesive No. 1469) which solidified on contact with body fluids. The latter method formed a stronger bond and was used from the beginning of April 1992 to the end of the data collection.

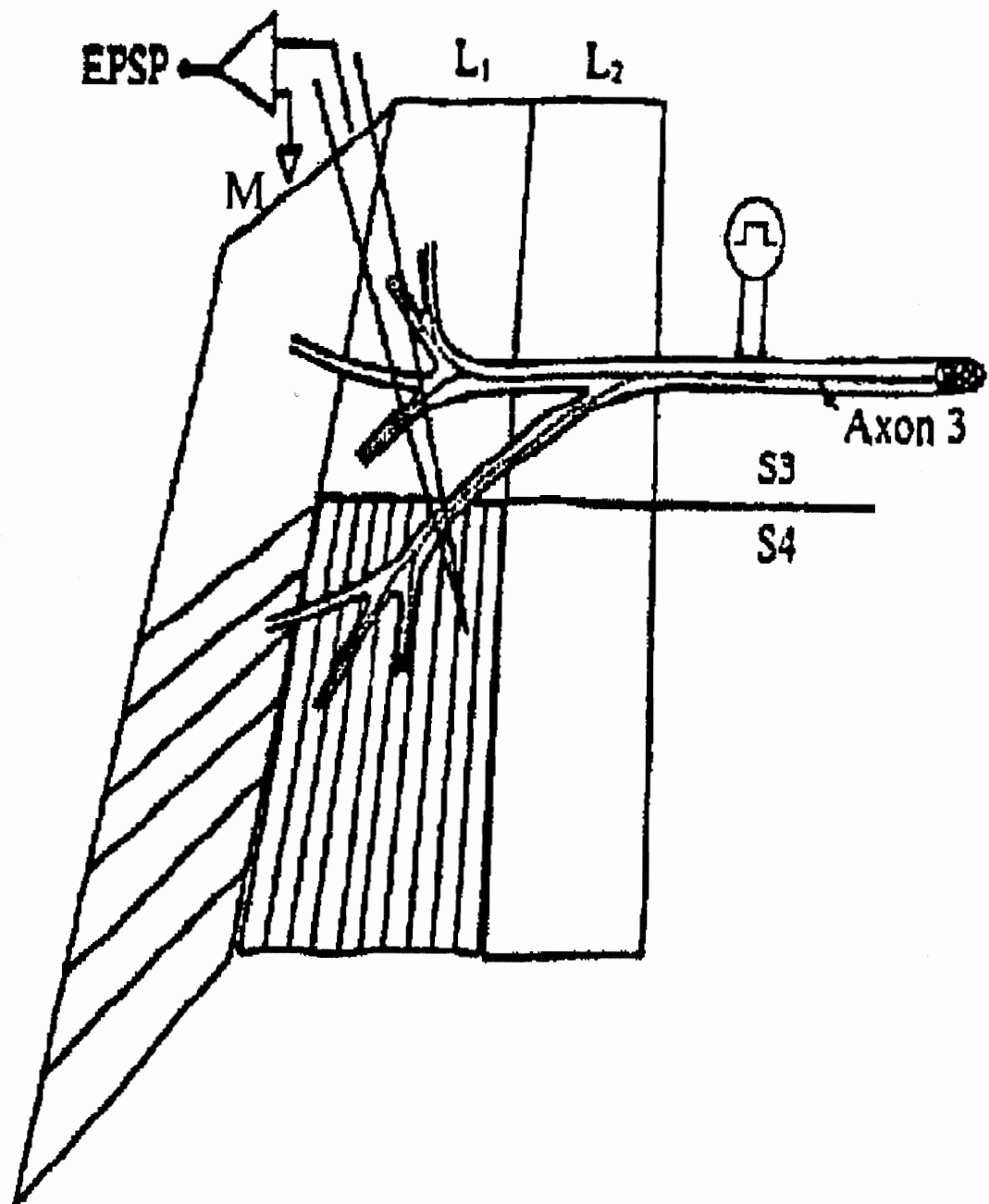
The wires in segment 3 were connected to an electronic stimulator and those in segment 4 were connected to a Grass P15 preamplifier which was used to monitor muscle potential. The stimulating voltage was then gradually increased until muscle potentials were observed from segment 4. The animals were then stimulated at this intensity using 1s bursts with 1s rest periods in between. The stimulating frequency within the bursts was 5Hz, giving an average frequency of 2.5 Hz throughout the conditioning session. The neurons were stimulated in this manner for 2 hours per day for 3 days. The voltage was increased if muscle potentials were lost from segment 4. Crayfish were kept in a 10°C cold room between conditioning periods and until they were used for intracellular recording, which occurred within 5 days of the last conditioning session. The procedure was modified after the beginning of May 1992. The same stimulus frequency, burst rate and burst duration were used in these later experiments, but the animals were stimulated for 4 hours per day, instead of 2h per day. The neurons were stimulated for 2h, followed by a 1h rest and then another 2h of stimulation. This was done over a three day period. The stimulation protocol was changed in this way, to produce a more substantial reduction in transmitter release.

Intracellular Recording.

Crayfish were placed on ice to decrease activity in the nervous system. After several minutes, they were euthanized by decapitation and destruction of the brain and thoracic ganglia using scissors. The abdomen was cut away and the abdominal extensor muscles were exposed. The dorsal shell containing these muscles, was pinned in a sylgard-bottomed dish containing saline (see below). The abdomen was cut to fit the recording dish (2 mm by 1.5 mm by .5 mm (1.5 ml)) which was placed into a copper cooling chamber which kept the saline bath between 9-13 °C. Temperature did not vary much in individual experiments. The mean temperature during a one hour period was $10 \pm 0.12^{\circ}\text{C}$, with only a 0.7°C change from the highest to lowest temperature.

The nerves to the M muscle were cut to prevent twitching which would dislodge the recording electrode. The nerve bundle coming into segment 3 was drawn up into a stimulating suction electrode. The suction electrode was connected to the stimulator through a Grass stimulus isolation unit (SIU5), which reduced the size of the stimulus artifact. An intracellular electrode was positioned so that it pierced a cell near the anterior end of the L1 muscle in segment 4 (figure 3).

Figure 3: The intracellular recording technique. The anterior cells of the L1 muscle were penetrated while a suction electrode attached to the nerve of segment 3 was being stimulated. Five penetrations were made starting at the lateral edge and working medially across the muscle.



Three different salines were used in this study (Table I). Nearly all experiments were performed using two salines containing low Ca^{2+} and high Mg^{2+} levels, compared to the physiological concentrations of these ions. This lowered EPSP amplitude sufficiently to minimize muscle contractions that would otherwise dislodge the recording electrode and/or damage the muscle cell. In one of these salines, Ca^{2+} and Mg^{2+} concentrations respectively were 50% and 500% of the levels in normal saline (Table I). In the other the Ca^{2+} and Mg^{2+} levels were 75% and 300% of normal. The latter saline was used for preparations having very small EPSP's on both conditioned (2-5 mV) and unconditioned (5-10 mV) sides. This was done to allow a sufficiently large signal for assessing the effects of BAPTA-AM.

The nerve in segment 3 sends only 2 axons to muscle L1 in the 4th abdominal segment (Parnas and Atwood, 1966). One is an inhibitory axon, which causes a small inhibitory postsynaptic potential (IPSP) in L1. The other produces a large excitatory postsynaptic potential. Low frequency stimulation (0.5 Hz) was applied to this nerve in order to produce a PSP (Post Synaptic Potential) in L1. Once a PSP was obtained it was tested to determine whether or not it was produced from the excitatory or inhibitory neuron. The EPSP (excitatory post synaptic potential) from the excitatory axon is larger and has a more rapid decay than the IPSP. In addition, stimulating the inhibitory axon has very little effect on the peak amplitude of the EPSP. One half hour after establishment of an EPSP, BAPTA-AM was applied. This allowed time for the preparation to adapt to the stimulus and to produce a fairly steady output.

Table I: composition of the three salines used. All chemicals were obtained from either Fisher or Sigma chemical companies.

	Normal Saline	50% Calcium; 500% Magnesium Saline	75% Calcium; 300% Mg ²⁺ Saline
NaCl	205 mM	200.65 mM	202.83 mM
MgCl ₂ •6H ₂ O	2.45 mM	12.3 mM	7.38 mM
KCl	5.3 mM	5.36 mM	5.3 mM
CaCl ₂ •2H ₂ O	13.5 mM	6.75 mM	10.13 mM
HEPES	5 mM	5 mM	5 mM
pH	7.4	7.4	7.4

The signal output from the oscilloscope was converted from analog to digital using an A/D converter (TL-1 DMA Interface by Axon Instruments). Digital signals were then fed into an IBM compatible computer (purchased from Computan Inc) with the aid of two programs obtained from Dr. Milton Charlton (University of Toronto). One programme (called ACQ) was used for acquiring data into files and a companion programme (named ANACQ) was used to measure the peak amplitude of the EPSP's. The early experiments were recorded using a VHS tape recorder (HQ SR1000), which was connected to a Neuro-corder DR-390 VHS adaptor, from Neuro Data Instruments Corp.

The data file produced by ANACQ (containing peak EPSP amplitudes) was imported into Lotus 123 for further analysis. EPSP's were corrected for non-linear summation using an equation developed by Martin, (1955), which takes into account the effect of transmembrane potential on the end plate potential. The equation is as follows:

$$EPSP' = (EPSP) / (1 - (EPSP / (V_m + 11.5)))$$

where EPSP is the peak amplitude of the excitatory post synaptic potential and V_m is the resting potential across the membrane and 11.5 reflects the reversal potential (Onodera and Takeuchi, 1975). The effects of BAPTA-AM and of conditioning were assessed using the adjusted value, EPSP'.

EPSP's were recorded from several sites in each muscle (see below), and at each site several responses were averaged. The means of the conditioned and control EPSP's were calculated by averaging the EPSP's produced prior to the addition of BAPTA-AM on the conditioned and control sides of the

animal. The equation used to calculate the % reduction in EPSP due to conditioning was:

$$\text{Effect of Conditioning} = (1 - (\text{mean Conditioned EPSP} / \text{mean Control EPSP})) * 100.$$

BAPTA Addition

The dose-response characteristics of BAPTA-AM were examined using unconditioned neurons. Isolated neuromuscular preparations were first perfused in 50% Calcium, 500% Magnesium Crayfish Saline for approximately 30 minutes. At this time the BAPTA-AM ((1,2-bis (2-amino phenoxy) ethane N,N,N',N'-tetraacetic acid - Acetoxy Methylester obtained from Molecular Probes Inc (catalogue # B-1205)) was applied. Stock solutions of 25 mM BAPTA-AM were prepared by dissolving each 5 mg aliquot from the manufacturer, in 1.34 ml of DMSO (Dimethylsulfoxide, Fisher Chemical). The stock solution was used to prepare experimental salines, in which the BAPTA-AM concentrations ranged from 5 μ M to 250 μ M. The recording chamber was perfused with saline containing BAPTA-AM for 40 min, at which time perfusion was switched back to saline without BAPTA-AM. In each experiment the effect of BAPTA-AM was determined by monitoring EPSP's from a single muscle cell before, during and after application of BAPTA-AM. This cell was always located toward the medial side and proximal end of the L1 muscle. The left side of the animal was used in 50% of the experiments and the right side was used in the other 50%.

Table II: Sources and abbreviations of the chemicals used.

Material	Abbreviation: catalogue no.	Source
[1,2-bis (2-amino phenoxy) ethane N,N,N',N'-tetraacetic acid]- Acetoxy Methylester	BAPTA-AM B-1205	Molecular Probes Inc. Eugene OR U.S.A.
[2'7'-bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein]- Acetoxy Methylester	BCECF-AM B-1170	Molecular Probes Inc. Eugene OR U.S.A.
Calcium Chloride	CaCl B10070	BDH Inc. Tor. Ont. Can.
DiMethylSulfOxide	DMSO D-5879	Sigma Chemical Co. St. Louis MO U.S.A.
N-[2-Hydroxyethyl] piperazine-N'-[2ethanesulfonic acid	HEPES H-3375	Sigma Chemical Co. St. Louis MO U.S.A.
Magnesium Chloride	MgCl M33-500	Fisher Scientific Fair Lawn, NJ U.S.A.
Sodium Chloride	NaCl S271-500	Fisher Scientific Fair Lawn, NJ U.S.A.
Sodium Hydroxide	NaOH R01419	BDH Inc. Tor. Ont. Can.
Potassium Chloride	KCl B10198	BDH Inc. Tor., Ont. Can.

A slightly different procedure was used to assess the effect of conditioning. EPSP's were recorded from both sides of the isolated abdomen for an initial period of 30 min while perfusing the chamber with BAPTA-AM free saline. Recordings were made from several sites along the L1 muscle, from the lateral to the medial side. 5 cells were penetrated, each recording site equidistant from the next. The first recording side was alternately, on either the conditioned or unconditioned half of the animal. The other side of the animal continued to be examined during the application of 25 or 50 μ M BAPTA-AM (40 min perfusion). Saline without BAPTA-AM was applied and EPSP's were recorded from five sites in approximately the same locations as pre-BAPTA-AM addition (again for a 30 minute period). The side of the first recordings was then resampled (5 sites, 30 minutes). The EPSP's at the time of the conditioning experiments were sometimes very small, even in unconditioned preparations (eg. Figure 9). Therefore, a mixture of normal saline and 50% calcium, 500% magnesium saline was used in a 1:1 ratio. This is how the 75% Ca^{++} and 300% Mg^{++} saline was produced that is shown in Table I.

50 μ M BAPTA-AM had a different effect at different times of the year (see below). This was probably not due to a bad batch of BAPTA-AM since more than one batch was used over the time of the study. There were occasions when BAPTA-AM was not effective (< 30% reduction in EPSP amplitude at 50 μ M). At these times the data were not included in the final results, the BAPTA-AM was thrown out and a new batch was used.

Saline and Ester Cleavage Controls

To test the effect of reduced entry of calcium into the terminal on the effect of BAPTA-AM, three salines with different calcium concentrations were used. Unconditioned preparations were perfused with 50% Ca^{2+} 500% Mg^{2+} saline, followed by 75% Ca^{2+} , 300% Mg^{2+} saline followed by normal saline. In each case, the full effect of the change in saline occurred in about 30 min. EPSP's were recorded from 5 sites over 30 min, for each saline. After the EPSP's were recorded in normal saline, 50 μM BAPTA-AM was added and allowed to perfuse the chamber for 40 min. The salines were then changed in the reverse order to determine the post-BAPTA-AM effect. This should simulate what would occur if the cause of LTA is reduced entry of calcium into the terminals.

To test the effect of the cleavage of the ester from BAPTA, the cell permeant pH indicator BCECF-AM (2'7'-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein-acetoxymethyl ester from Molecular Probes Inc. (cat# B-1170) was used. A concentration of 100 μM was added to a preparation using the same methodology as was used for BAPTA-AM with one modification. DMSO was added to the initial saline as well as the BCECF-AM saline; this made it possible to separate the effect of the AM cleavage from the effect of DMSO on EPSP amplitude.

RESULTS:

Effects of BAPTA

BAPTA-AM decreases the amplitude of evoked EPSP's in muscle L1. Figure 4 depicts the time course of the effect of 50 μ M BAPTA-AM on a representative preparation. In the example shown, perfusion of the recording chamber with BAPTA-AM causes a gradual decline in EPSP amplitude over 20-30 min, until its maximal effect is realized. The time required for the maximal effect may reflect: i) the time it takes for BAPTA-AM to cross the membrane; ii) conversion to the active salt form by esterase; iii) time to reach equilibrium within the nerve terminals; iv) the time for turnover of fluids in the recording dish. Once the full effect of BAPTA-AM has been achieved, there is no obvious change in EPSP for at least 40 min (data not shown). This suggests that BAPTA is not leaving the nerve terminals, nor is it being degraded in any observable way. Representative EPSP's recorded before the application of 50 μ M BAPTA-AM and after its full effect are shown in Figure 5. In this case, BAPTA-AM caused a 33% reduction in EPSP amplitude.

Figure 4: Time course of the effect of BAPTA-AM on EPSP amplitude. 50 μ M BAPTA-AM had its full effect 20-30 min after being applied to the preparation. Subsequently the EPSP did not dramatically change in amplitude. These data were collected from a single preparation.

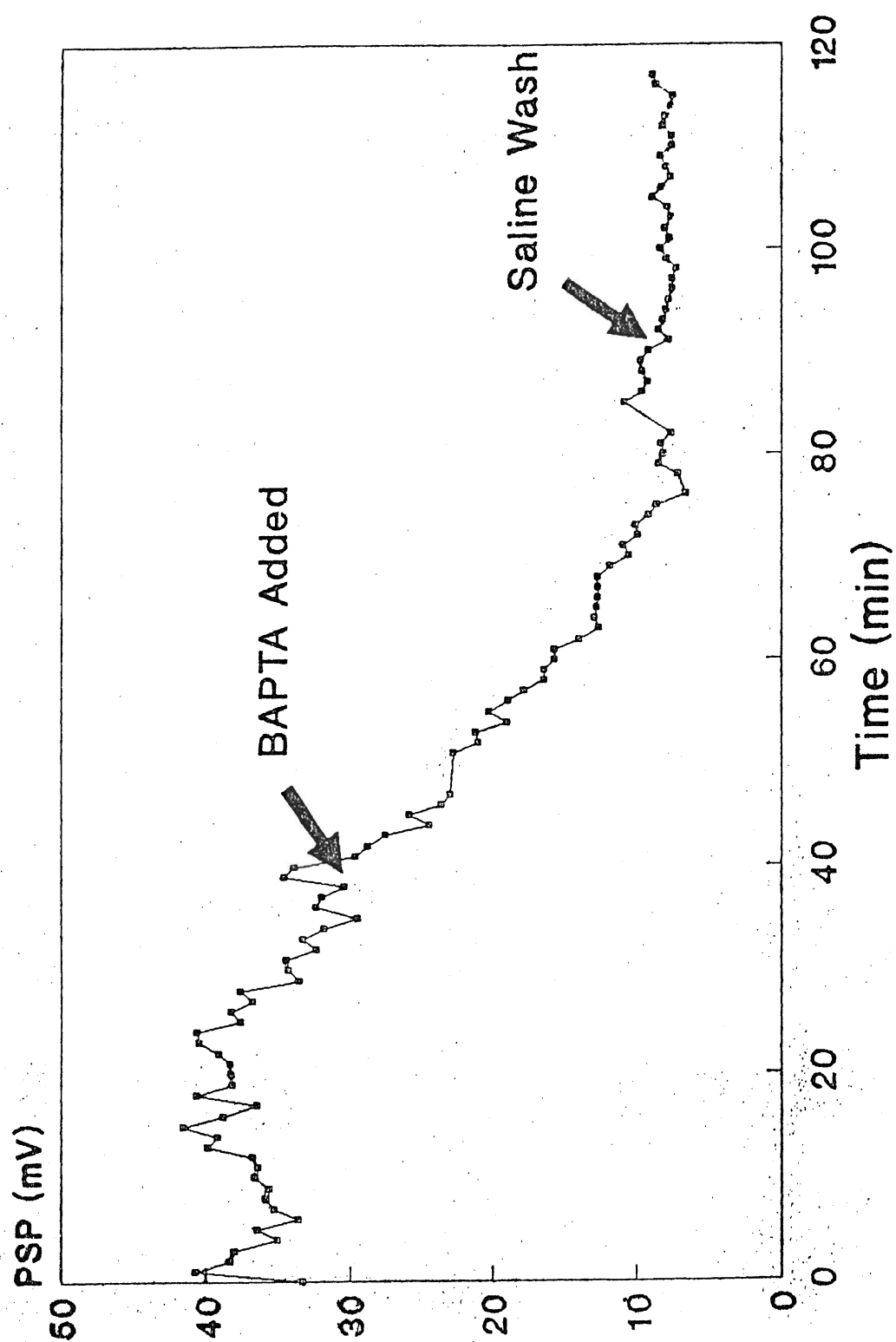
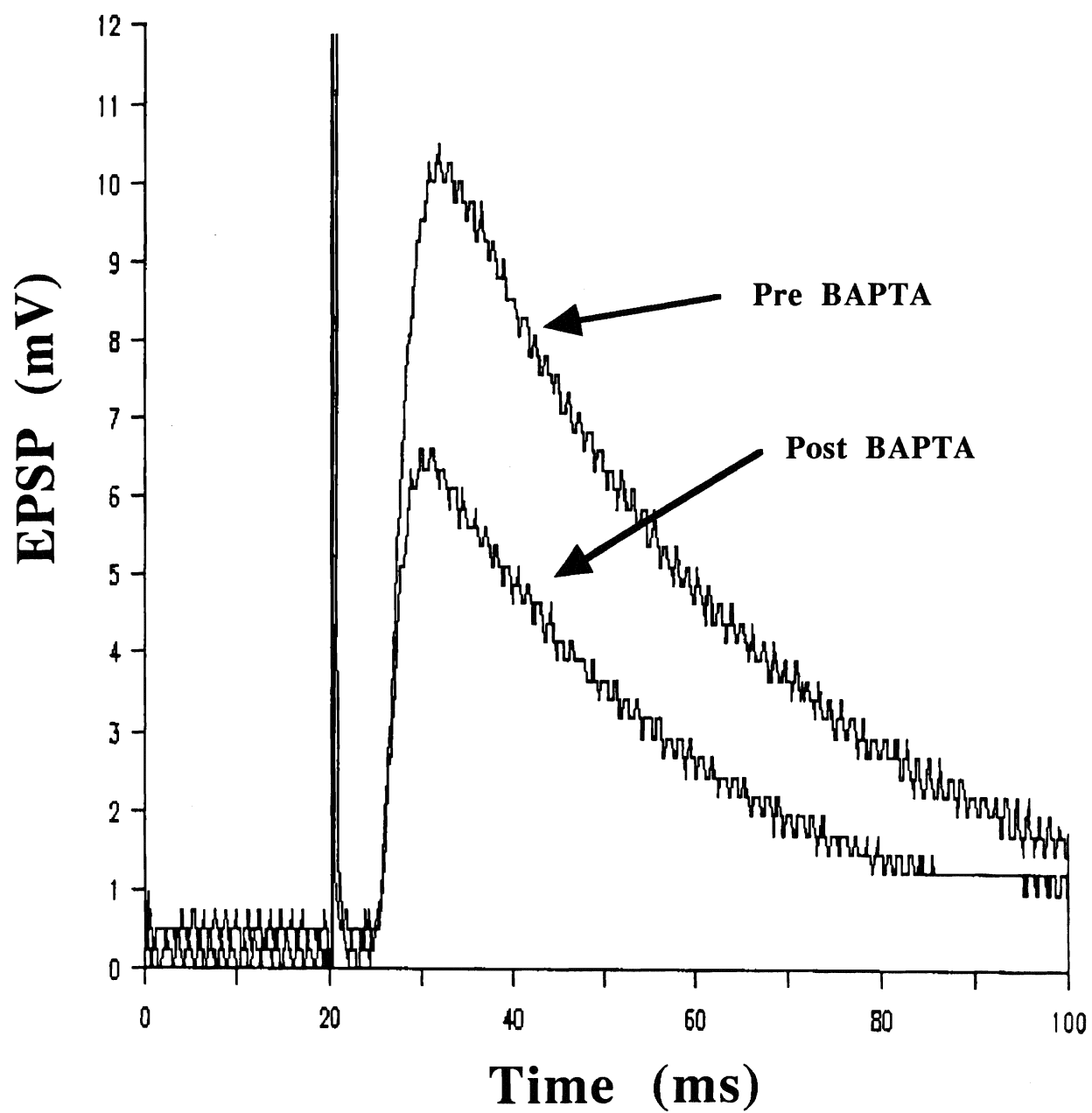


Figure 5: Sample EPSP's showing the effect of 50 μ M BAPTA-AM. This figure shows signal averaged EPSP's prior to the application of BAPTA-AM (pre) and after 40 min of exposure to BAPTA-AM (post). In this case BAPTA-AM caused a 33% drop in EPSP amplitude. Each EPSP represents the average of 4 responses.



The effect of BAPTA-AM on EPSP amplitude is concentration dependent (figure 6). The relationship between the reduction in EPSP amplitude and the log of the BAPTA-AM concentration resembles a saturation curve up to approximately 50 μM . Maximal inhibition is observed at 50 μM which decreases EPSP amplitude by approximately 78%. At 100 μM , however, BAPTA-AM is less effective (by 35%). This may not be due to BAPTA-AM *per se* but to the solvent DMSO and cleavage of the AM group. In these experiments the BAPTA-AM concentration was increased by adding greater volumes of a stock solution containing DMSO. Thus, the concentration of DMSO increased with that of BAPTA-AM. DMSO (0.4% v/v) causes a large increase in transmitter release (figure 6B) which could mask the effect of BAPTA-AM. Another factor that contributes to the decline in inhibition appears to be the ester cleavage of BAPTA-AM, which produces formaldehyde and acetic acid. The control experiment with BCECF-AM showed an effect of the ester cleavage on EPSP's. The EPSP amplitude increased by $16.5 \pm 2\%$ after the addition of a pH indicator (BCECF) with an AM linkage (Figure 6B). This indicates that high concentrations of an AM linked probe can oppose the effect of the calcium chelator, BAPTA. A final explanation is that there may be toxic effects from high concentrations of the salt form of BAPTA within the cell, but this possibility was not tested in this thesis.

Effects of conditioning.

Some effects of conditioning were manifest during the *in vivo* stimulation procedure. Stimulation of segment 3 *in vivo* caused an increase in the electromyogram (EMG) amplitude on the second day (Figure 7). This might be due to long-term facilitation (Lnenicka and Atwood, 1985a; Sherman and Atwood, 1971). On the third day of stimulation, a much smaller EMG was observed, reflecting LTA at the neuromuscular synapses.

EPSP's were recorded in isolated neuromuscular preparations between 1 and 5 days after the end of *in vivo* conditioning. The EPSP's in muscle L1 were evoked by stimulating the nerve at a low frequency (0.5 Hz) in order to minimize both facilitation and depression. This frequency of stimulation was used for all experiments. Such stimuli were delivered as a brief train of 4 impulses, with 52 s between each train. Sample EPSP's are shown in Figures 5 and 8.

Figure 6: Dose-dependent effects of BAPTA-AM on EPSP's. Part A shows a concentration effect curve for BAPTA-AM (n=5 preparations per point). A possible explanation for the reduction in inhibition seen at 100 μ M was examined in Part B. This part shows that 0.4% DMSO (v/v) (the highest concentration of DMSO used, and the concentration used for the 100 μ M trials) increases EPSP amplitude (n=6). Cleavage of the AM group also increases EPSP amplitude and is shown beside the DMSO effect (n=5).

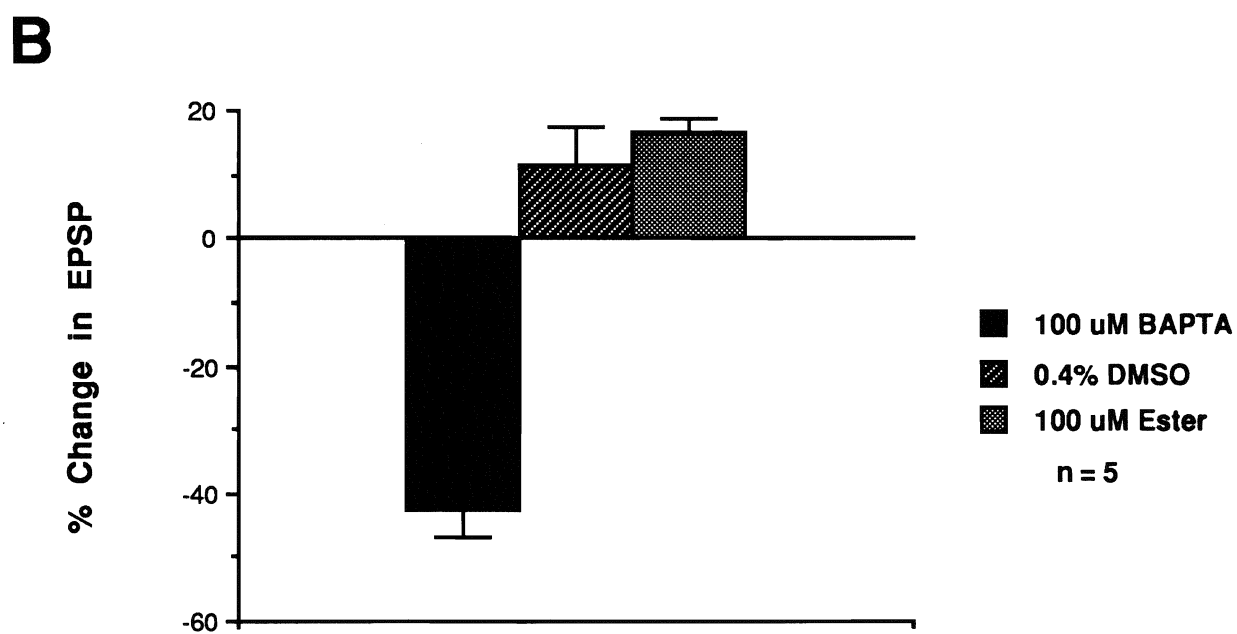
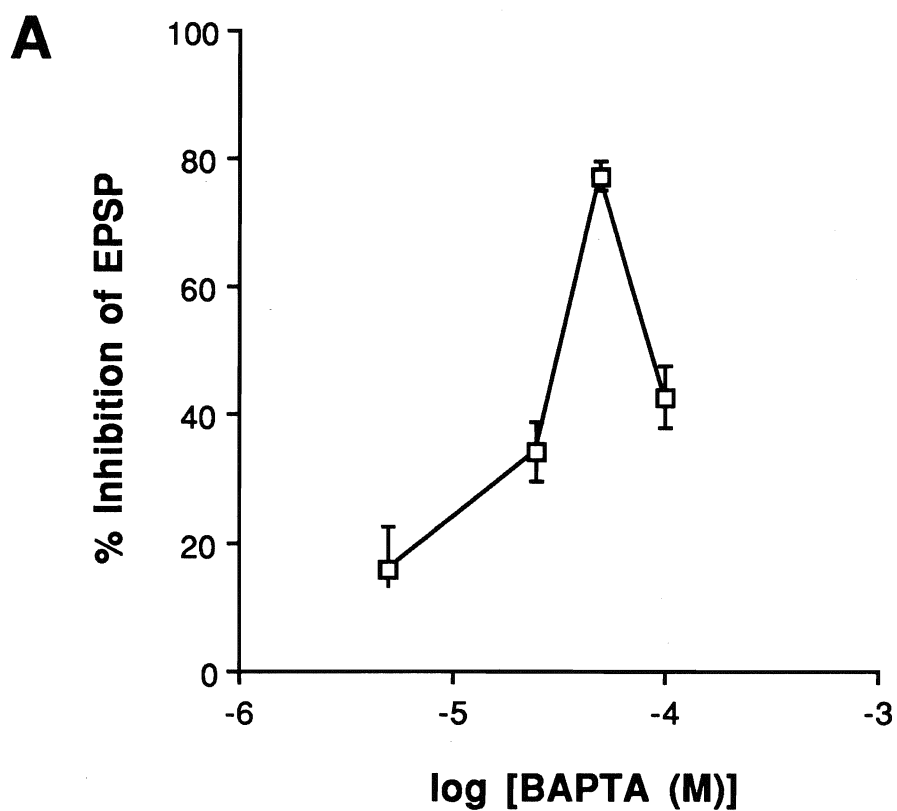
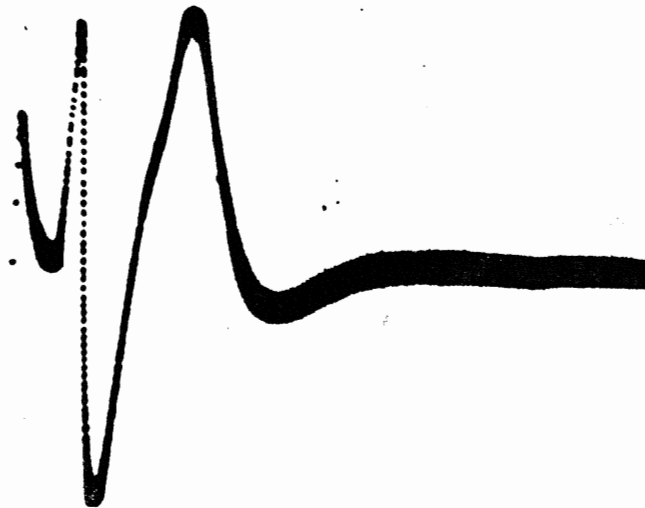
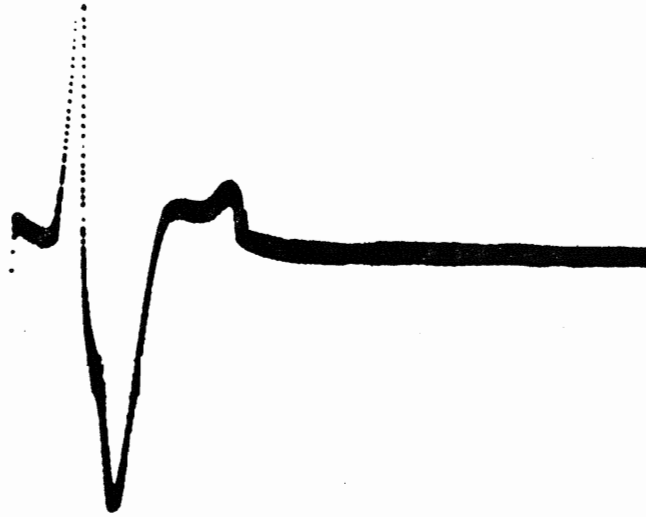


Figure 7: EMG's from each day of stimulation. The top, middle and bottom traces, respectively, are recordings made on the first, second and third days of *in vivo* stimulation. LTA is apparent by the 3rd day of stimulation.



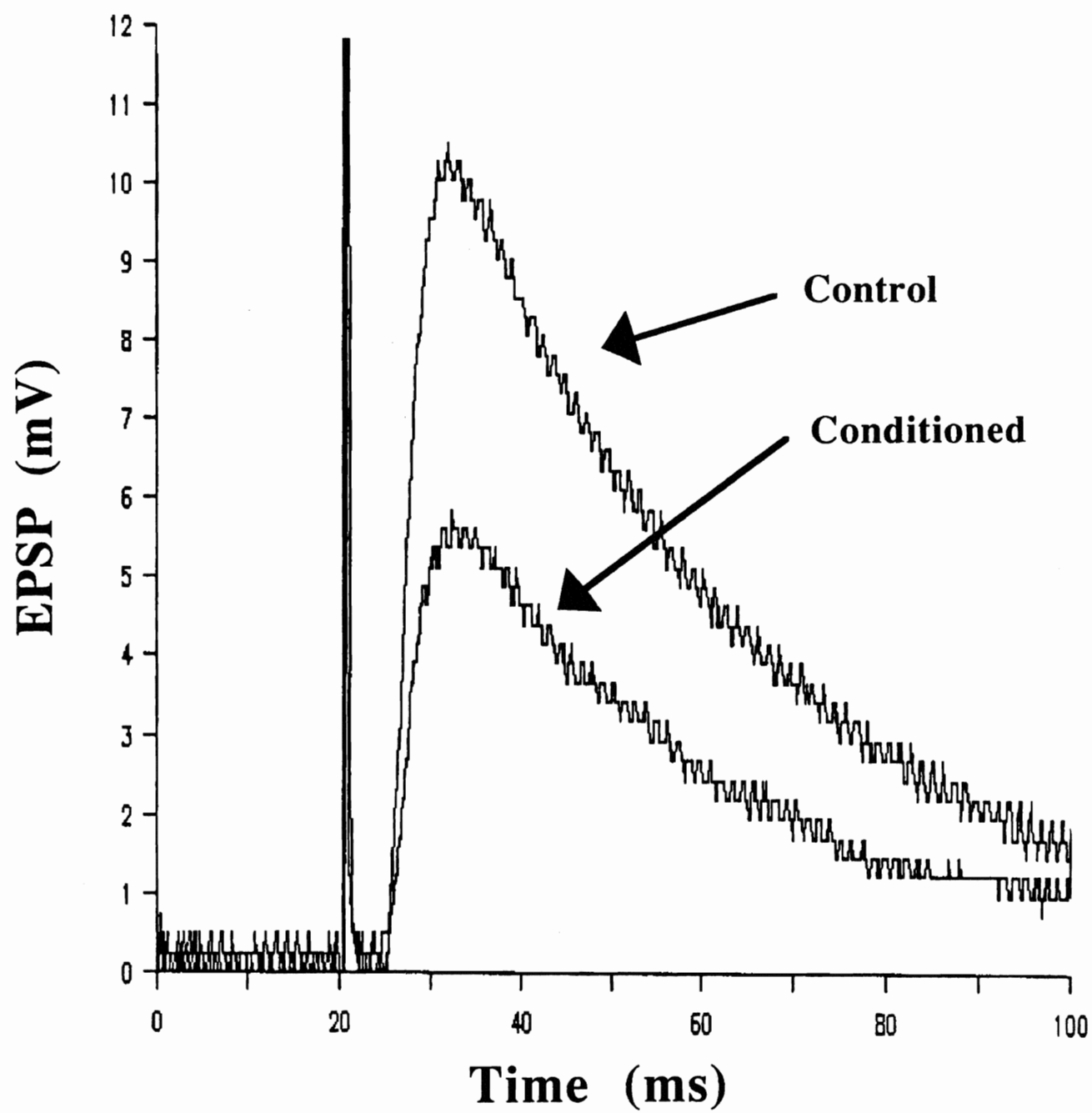
1 mV
10 ms



Other studies (Bradacs *et al*, 1991; Lnenicka and Atwood, 1985 a,b; Mercier and Atwood, 1989) have examined synaptic fatigue by determining the reduction in EPSP amplitude during repeated stimulation at 5 Hz. Fatigue, which is known to be reduced by conditioning, was not examined in this study.

Conditioning was effective at reducing the amplitude of EPSP's (Figure 8). Conditioning does not alter the percentage reduction in EPSP's induced by BAPTA-AM (Figures 9 and 10). The EPSP's measured on the control side prior to the addition of BAPTA-AM are much larger than the EPSP's on the conditioned side of the animals. The mean on the control side (20.76 ± 3.76 mV) is significantly larger ($t=4.91$ and $p<0.001$) than the mean on the conditioned side (9.56 ± 1.74 mV). Thus the conditioning process successfully induced LTA. Sample EPSP's, showing the effects of conditioning and of BAPTA-AM, are shown in Figures 8 and 5. BAPTA-AM causes a significant decrease in the amplitude of the EPSP's on both the conditioned ($t=2.85$ and $p<0.02$) and control ($t=4.4$ and $p<0.01$) sides of the animal. However, the responses varied among different preparations. Figure 12 shows the effect of conditioning (expressed as the percent reduction in EPSP's) plotted against the difference in the effect of BAPTA-AM on the control side versus the conditioned side of the animal. In nine animals, conditioning reduced EPSP amplitude by more than 40%. Of these animals, 4 showed a larger effect of BAPTA-AM on the control side, 2 showed a larger effect of BAPTA-AM on the conditioned side, and 3 showed little or no difference between the two sides.

Figure 8: Sample EPSP's showing the effect of conditioning on EPSP amplitude. The top trace shows the control EPSP and the bottom trace is taken from the conditioned side of the same animal.



Seasonal Variation

EPSP amplitude appeared to show seasonal variability during the 11 month period of this study. EPSP's were consistently small from January through March, 1992 and July through August, 1992 but they tended to be larger and more variable from October through December, 1991 and April through June 1992 (Figure 9). Lnenicka and Zhao, (1990) also observed seasonal variability in the EPSP's of the crayfish claw closer muscle, which they correlated with susceptibility to conditioning. In their studies, LTA was exhibited less in seasons with small EPSP's than in seasons with large EPSP's. A possible correlation between the initial EPSP amplitude and the effect of conditioning was examined (figure 13) in order to compare this work with that of Lnenicka and Zhao, (1990). There were differences in stimulus frequency (used in conditioning the animals) and calcium concentrations among individual experiments in the present study. Therefore, correlations were calculated independently for each group of data. No significant correlation was observed when the animals were conditioned for 2h/day ($R=0.76$; $df=4$), when conditioned for 4h/day in 50% calcium ($R=0.65$; $df=4$) nor 75% calcium saline ($R=0.68$; $df=3$) (Figure 13). There were low degrees of freedom with each of these data sets. Therefore a quantitative analysis of the differences between

Figure 9: Seasonal variation in EPSP amplitudes. EPSP's were recorded from the extensor muscle prior to the addition of BAPTA-AM, and are shown for the months over which experiments were performed. EPSP's were higher in the fall and spring than in the winter and summer months. All values were obtained while perfusing with 50% calcium and 500% magnesium saline. Each value represents the mean of 5 recording sites from 1 preparation.

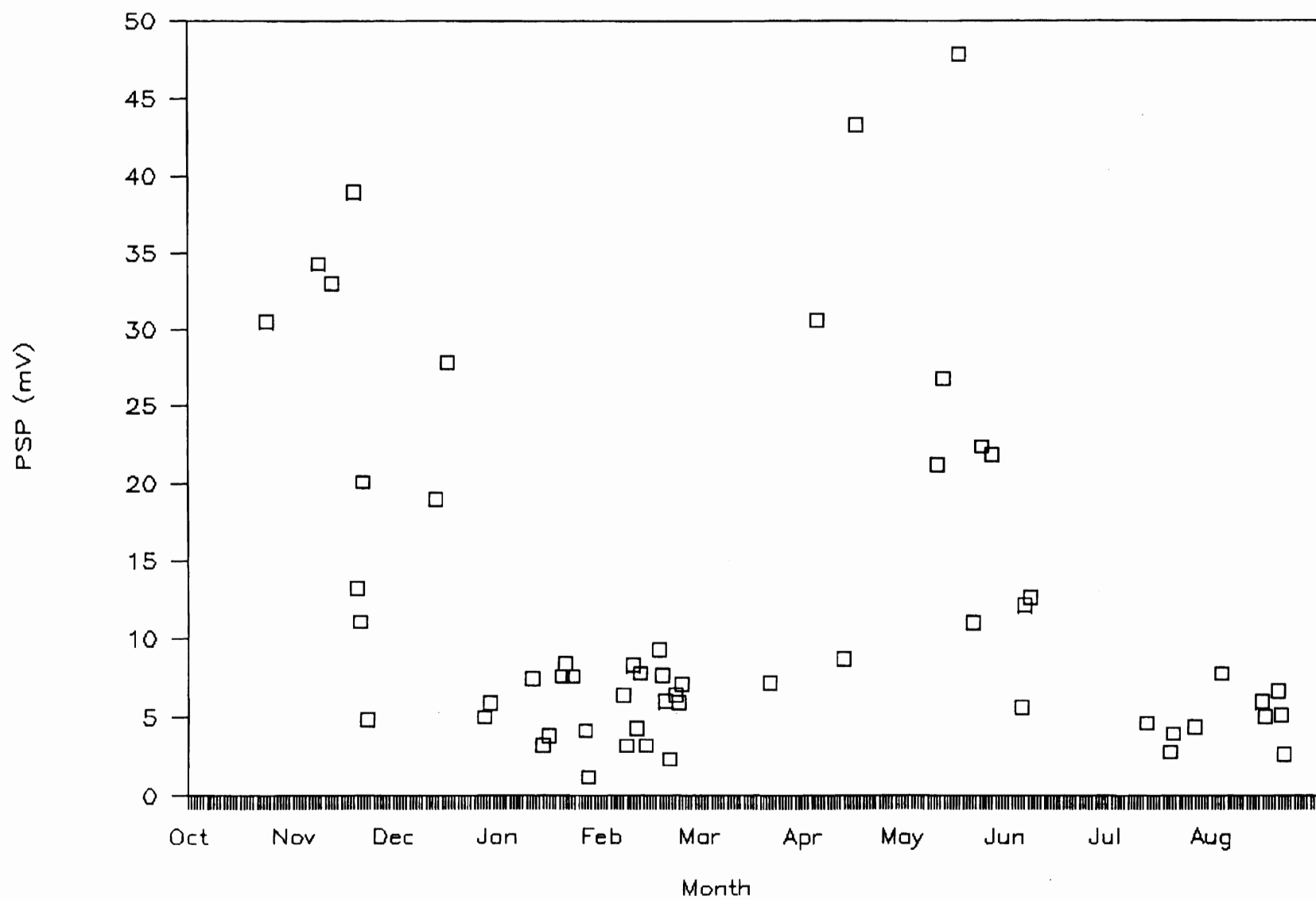
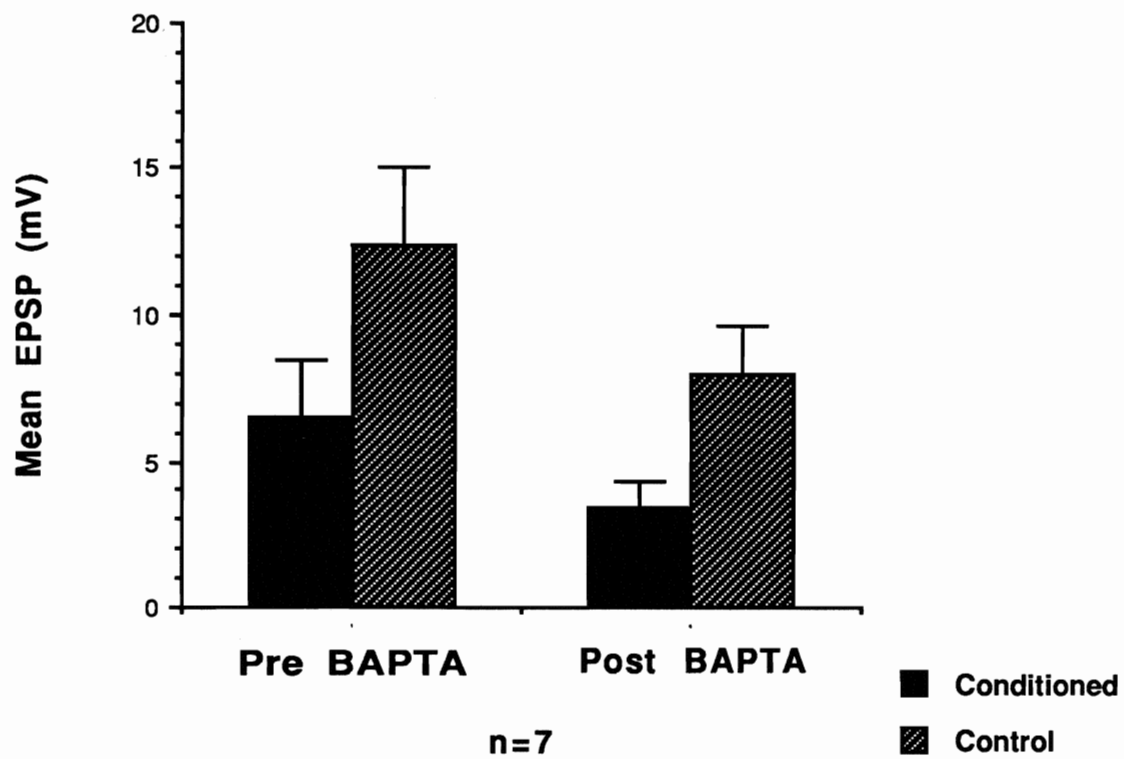
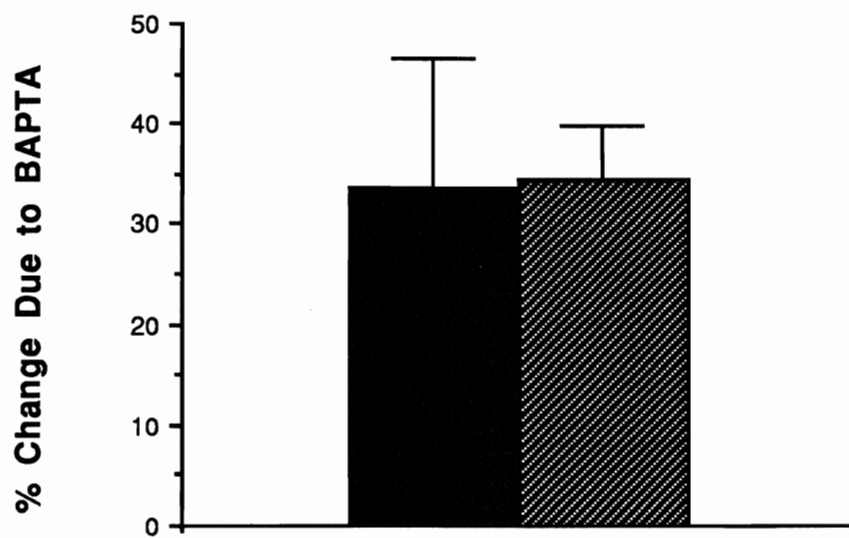


Figure 10: Effect of of BAPTA-AM on amplitudes of EPSP's in control and conditioned terminals. A: EPSP amplitude pre and post addition of BAPTA-AM and B: % decrease in EPSP due to BAPTA-AM (25 μ M). All experiments were performed using 50% Ca^{2+} 500% Mg^{2+} saline.

A**B**

2h/day conditioning and 4h/day conditioning cannot be done. Lnenicka and Zhao, (1990) observed seasonal decreases in EPSP amplitude in the crayfish claw, which does not show LTA after a certain age. This is different from the extensor muscle of the tail, which does show LTA in older animals (Mercier, 1991). The contrast in both seasonality and susceptibility to conditioning in the claw of the animal when compared to the tail, could represent a basic difference in physiology of these two neuromuscular systems.

50 μ M BAPTA-AM was used throughout the year, and yielded different results at different times of the year. When the dose response curve was determined (Oct-Jan), 50 μ M BAPTA-AM caused a $77.18 \pm 2.94\%$ reduction in EPSP amplitude. When the conditioning studies were performed (Apr-Aug), the effect (a $56 \pm 3.83\%$ reduction in EPSP amplitude) was significantly lower ($t=2.73$; $p=0.023$).

Effects of Calcium/Magnesium Ratio

To test the effect of external calcium concentration on transmitter release and also the effect of cleavage of the AM ester, control experiments were performed. The calcium concentration controls were performed to measure the effect of using two salines with differing calcium and magnesium concentrations.

The saline controls showed no significant difference in the effect of BAPTA-AM with 50% Ca^{2+} 500% Mg^{2+} saline when compared to 75% Ca^{2+} 300% Mg^{2+} ($t=1.04$ and $p>0.2$) nor was there a difference between the 75% Ca^{2+} saline and Normal saline ($t=2.54$ and $p>0.05$). There was however a significant ($t=4.11$ and $p>0.01$ two tailed student t test) difference between the 50% Ca^{2+} 500% Mg^{2+} and normal salines. There was no significant difference in the effect of conditioning ($t=0.34$ and $p>0.2$) when using the two salines (Figure 14). For these reasons the data collected using the two salines were pooled.

Figure 11: Effect of of BAPTA-AM on amplitudes of EPSP's in control and conditioned terminals. A: EPSP amplitude pre and post addition of BAPTA-AM and B: % decrease in EPSP due to BAPTA-AM (50 μ M). Five of the experiments were performed using 75% Ca^{2+} 300% Mg^{2+} and 6 were performed using 50% Ca^{2+} 500% Mg^{2+} salines. Since there was no significant difference between the effects of the salines, the two were pooled.

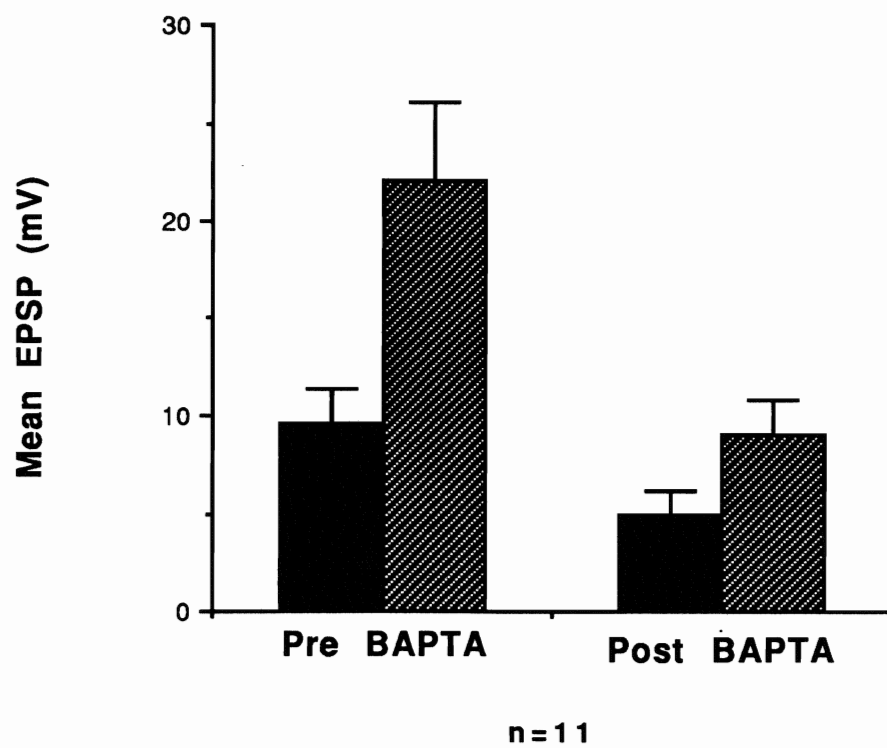
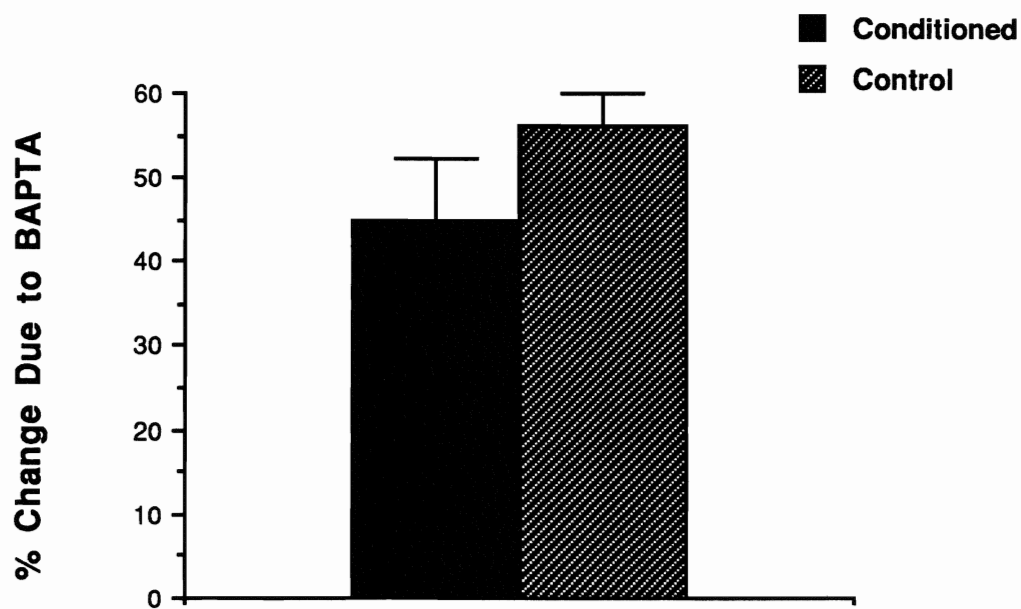
A**B**

Figure 12: Relationship between the effectiveness of BAPTA-AM and LTA. The difference in effect of BAPTA-AM on the control verses the conditioned side of the animal plotted against the effect of conditioning (expressed as percent reduction in EPSP amplitude).

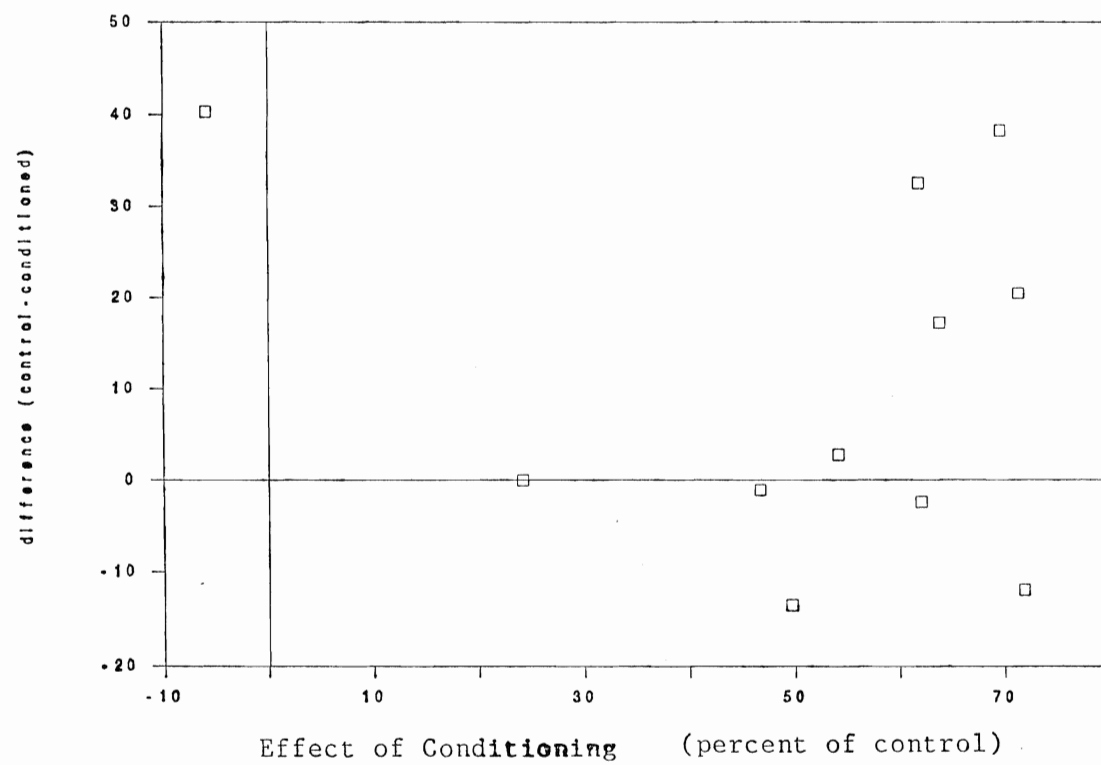


Figure 13: Effect of conditioning plotted against initial amplitude of EPSP. Square symbols represent experiments performed using 50% Ca^{2+} -500% Mg^{2+} saline and conditioned for 4h/day. Diamonds represent experiments performed using 50% Ca^{2+} -500% Mg^{2+} and conditioned for 2h/day. Plus symbols represent experiments performed using 75% Ca^{2+} -300% Mg^{2+} saline and conditioned for 4h/day.

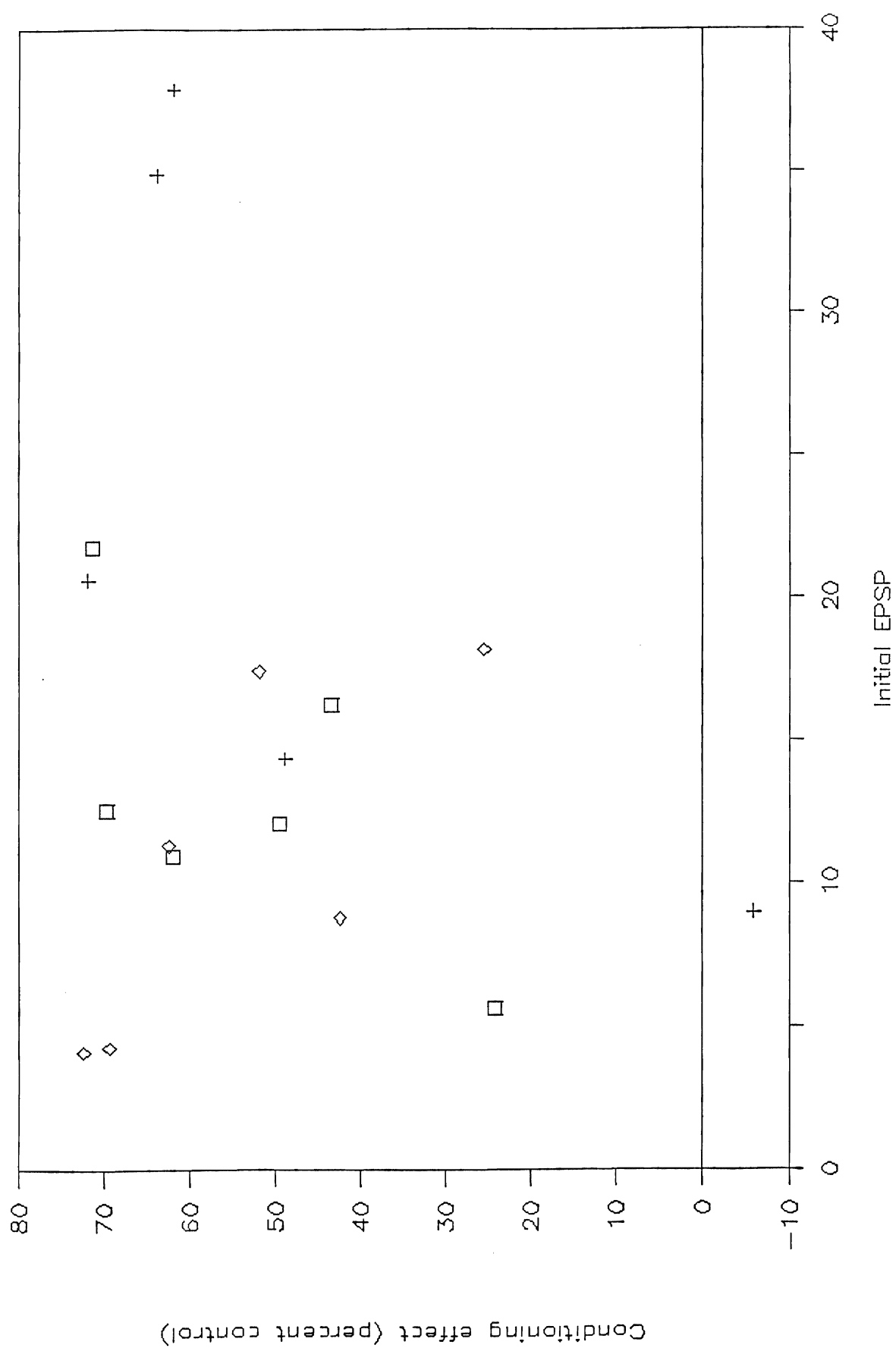
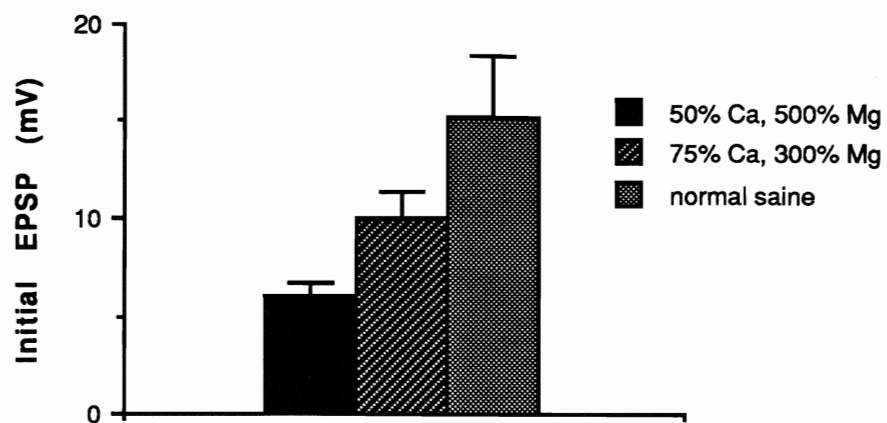
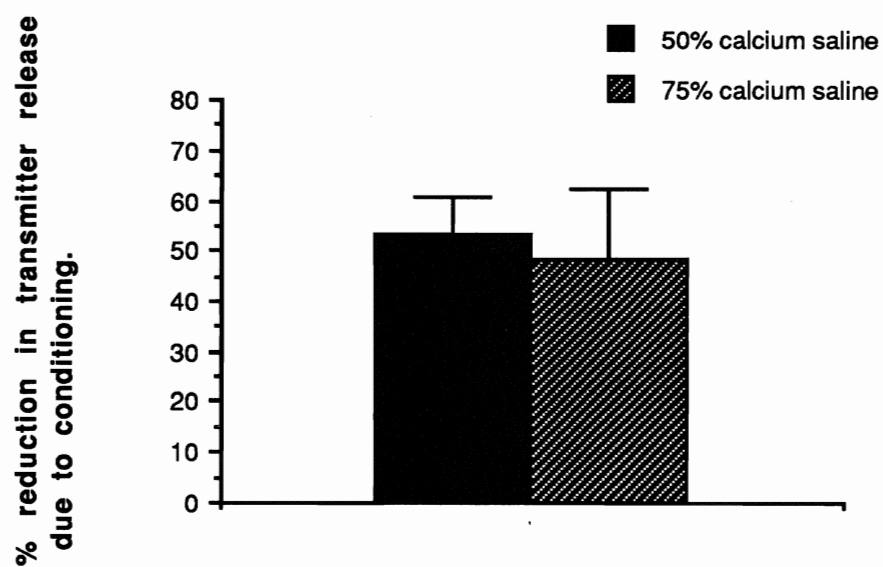
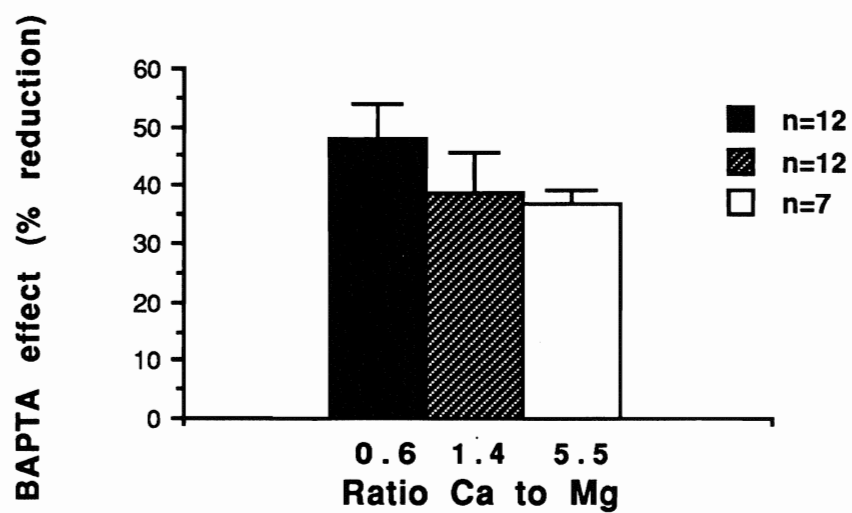


Figure 14: The effect of the various salines on initial EPSP amplitude (A), on conditioning (B) and % reduction in EPSP amplitude due to BAPTA-AM (C). Only unconditioned animals and the control side of conditioned animals were compared. There was no significant difference between the two salines used in this study in B or C.

A**B****C**

DISCUSSION:

In this study LTA was observed in one of the abdominal extensor muscles of crayfish. The effects observed in the extensor muscle ($56.8 \pm 8.9\%$ reduction in EPSP amplitude) were comparable to results obtained in the crayfish claw closer muscle (Lnenicka and Atwood, 1985 a,b) and those observed in adult crayfish extensor nerves stimulated over a longer period of time (Mercier and Atwood, 1989). Fatigue resistance should increase in the conditioned neurons (Lnenicka and Atwood, 1985 a,b) but was not studied in this thesis.

The central hypothesis of this thesis is that the reduction in transmitter release during LTA is caused primarily by changes in intrinsic calcium buffering in the nerve terminals. Experiments showed that there was no significant difference between effects of BAPTA-AM on conditioned and unconditioned phasic neurons ($t=1.87$ and $p>0.05$, $50\ \mu\text{M}$ BAPTA-AM; $t=0.07$ and $p>0.2$, $25\ \mu\text{M}$ BAPTA-AM). This suggests that a change in intrinsic calcium buffering capacity is not the major factor responsible for LTA. The results do not show that intrinsic buffers were unchanged by LTA, only that the changes in buffering were not observable with the technique used. This could be due to the intrinsic calcium buffers not being as mobile as BAPTA, not having the same affinity for calcium as BAPTA, nor being as rapid at binding calcium as BAPTA. If any of these were the case, a change in intrinsic calcium buffering would not be observed. Changes in intrinsic calcium buffers may assist in LTA

effects, by interacting with various factors responsible for release (eg. Ca^{2+} channels, vesicles, transmitter release sites). Such changes, while not observable, would still have a big impact on reduction in transmitter release during LTA.

Experiments showed no significant difference between the effect of BAPTA-AM in either of the low calcium salines. This shows that the experiments performed in the two different salines were not significantly affected by the different calcium concentrations. The observation that BAPTA-AM has the greatest effect at the lowest calcium concentration (compared to normal saline) suggests that reduced calcium entry may mask the full effect of BAPTA-AM on this experimental preparation. Therefore, an experimental protocol that examines both calcium entry and calcium buffering should be originated in order to examine the cause of LTA in greater detail.

Further experiments to see how much the buffering capacity does change could and should be done. One possible experiment is to use even lower levels of calcium in the perfusion saline. In this way the ability of the intrinsic buffer to bind calcium would not be overwhelmed by the incoming calcium signal. If BAPTA-AM was then applied in a similar manner as in this thesis, differences in intrinsic buffers associated with LTA may be more easily observable. These experiments should to be done at times of the year when EPSP amplitudes are high. This would help to keep the EPSP signal above background noise when BAPTA-AM is added in low calcium saline. Another set of experiments could be to use fluorescent Ca^{2+} probes in conjunction with high resolution

microscopy (such as Confocal Imaging) to see changes inside the nerve terminals as calcium enters.

An alternative to the hypothesis of this thesis is that there is less calcium actually getting into the terminal. This could be due to (1) modification of the calcium channels so that they either allowed less calcium in or closed after a shorter period of time and (2) degradation or removal of some of the calcium channels. Either of these occurrences would also decrease EPSP amplitudes. Such changes in calcium influx may cause no difference in the observable effect of BAPTA-AM on the conditioned as opposed to the control side of the animals studied. They may, however, mask the full effect of the change in intrinsic buffer. In the saline control experiments of this thesis, the effect of BAPTA-AM on transmitter release depends on external calcium concentration. This is opposite to what is hypothesized to happen if intrinsic calcium buffer was increasing. The two working in opposition would mask the changes in intrinsic calcium buffer. These two processes occurring simultaneously would make it difficult using the experimental protocol developed for this thesis, to observe the actual changes in intrinsic calcium buffering. Thus, we cannot exclude the possibility that LTA involves both a reduction in Ca^{2+} influx and increased buffering. However, the data suggest that the reduction in transmitter release during LTA is not solely due to a change in intrinsic calcium buffering.

The second possibility listed above (ie. calcium channel degradation) is suggested by the work of Franklin *et al*, 1992, who found that prolonged

depolarization of a cell results in long-term reduction of calcium currents. They concluded that the decrease in functional calcium channels was due to the net loss of channels rather than an increase in membrane surface area or some other factor. They further hypothesized that increased degradation of calcium channels caused the loss of current density. Further experiments comparing calcium currents in adapted neurons as compared to unadapted neurons could be done. Effects of proteases known to degrade membrane proteins could also be examined. Along the same line of reasoning, protease inhibitors could be studied to see if they inhibit LTA. A candidate protease in vertebrates is Calpin, which has been found to cause irreversible Ca-current 'rundown' in Guinea pig myocytes (Belles *et al.*, 1988) and to degrade a number of membrane proteins (Dice, 1987; Melloni and Pontremoli, 1989).

Another hypothesis is that intracellular calcium is having less of an effect on the release of transmitter. For example, calcium might be less able to effect the binding of vesicles at the release site. If this were the sole factor reducing transmitter release during LTA, BAPTA-AM would buffer out the same amount of calcium (and be equally effective) on the conditioned and the control sides. This possibility cannot be ruled out from the results obtained.

Toxicity of BAPTA-AM.

The literature supplied by Molecular Probes indicates that when BAPTA-AM enters a cell, the ester is cleaved by naturally occurring esterases. The major byproducts of this cleavage is formaldehyde and acetic acid, both

of which have already been shown to be toxic to cells (Alberts *et al*, 1983).

If too much formaldehyde or acetic acid is present in the cell (due to ester cleavage of large quantities of BAPTA-AM), transmitter release from the terminal could be affected. The results of the AM control experiment lend support to the hypothesis that too much ester cleavage effects the amplitude of the evoked EPSP. Application of another compound containing the same ester (AM) increased the EPSP amplitude by 20%. One possible explanation for this observation is that cleavage of the ester alters the pH in the terminals due to the production of acetic acid. A change in pH might affect the release of transmitter. Drapeau and Nachshen (1986) found that a decrease in pH intraterminally increased dopamine release by approximately 20 fold. Cohen and Van Der Kloot (1976) showed that lowering pH in frog neuromuscular terminals resulted in an increase in miniature end plate potentials. Another conjecture is that acetic acid stimulates an increase in ATP production in the mitochondria (Nicholls, 1982). How ATP effects transmitter release is however not clearly understood at this time.

BAPTA, Effect on Neuromuscular Synapses

The 25 μ M concentration of BAPTA-AM is the same as that used by Robitaille *et al* (1990) to study the frog neuromuscular junction. There was the same decrease in transmitter release (approximately 40 %) in both instances. The time course for the full effect of BAPTA-AM to occur is also 20-30 min in the frog neuromuscular junction. A major difference between the two

studies is that no transient increase in EPSP amplitude was observed in the crayfish deep extensors. Robitaille *et al* (1990) hypothesized that the increase they observed was due to the removal of calcium from calcium-activated potassium channels. If such channels are not activated, the action potential will be broadened and therefore more transmitter will be released. The same type of channel may not be present in the crustacean system.

Effects of DMSO.

DMSO was found to partially mask the effect of BAPTA, by increasing the amplitude of evoked EPSP's at a concentration of 0.4% v/v. Because of this, the concentration of DMSO was kept to minimal levels during the experiments where BAPTA-AM was used to study the effects of conditioning on intrinsic calcium buffering. The concentrations used were 0.2% v/v (50 μ M BAPTA-AM) and 0.1% v/v (25 μ M BAPTA-AM). We did not examine the effect of these concentrations alone on EPSP amplitude. Since both the conditioned and control sides of the animal were bathed in the same concentration of DMSO, masking effects of DMSO on BAPTA-AM should be the same on both sides. This should not affect the main conclusions of the study. The reason that DMSO increases transmitter release is not presently known.

Seasonal Variation

50 μ M BAPTA-AM was used throughout the year, and yielded different results at different times of the year. This difference could be a seasonal effect related to changes in intrinsic calcium buffers within the nerve terminals. What may be happening is that during spring and summer months the buffers may increase in quantity or in rate of chelating calcium. It may also be due to differences in esterase activity or concentration. If there is differential activity of esterases over the year, then there will be differing concentrations of BAPTA-AM within the nerve terminal over the year.

The finding of decreased EPSP amplitude in the extensor muscle, during both winter and summer months differs from seasonal changes in crayfish closer muscle reported by Lnenicka and Zhou, (1991). They found that EPSP's were smallest throughout the mid summer months and were consistently large throughout the winter months. However, work done on the frog (Pawson and Grinnell, 1989) showed that the lowest EPSP amplitudes were in the winter months. To determine whether there are seasonal changes in calcium buffering, studies using a single BAPTA-AM concentration would have to be done over an entire year. Such studies would have to be done for at least 3 years to see significant seasonal variability. In this way if it was observed that BAPTA-AM had less of an effect at one time of year over another, it would be suggestive of changing calcium regulation.

Calcium's role in LTA.

The notion that calcium may play a role in LTA stems from the fact that intracellular calcium plays a critical role in transmitter release. Thus, factors that determine free intracellular calcium levels, such as calcium entry and intracellular buffering, could be sites for long-term regulation of transmitter release.

Extracellular calcium has been found at the neuromuscular junction to be related to transmitter release by a log relationship of 3.9 (Dodge and Rahamimoff, 1967). Later work by Augustine *et al*, 1985 on the squid giant synapse, showed that there was a third power function relationship between presynaptic calcium current and post synaptic current. In addition Augustine and Charlton, (1986) found that post synaptic currents were high-exponent power functions of Ca^{2+} current regardless of whether Ca^{2+} current was modified by changes in membrane potential or changes in external Ca^{2+} concentration. From their results, they conclude that the 'co-operative' action of Ca^{2+} probably occurs at a step beyond the entry of Ca^{2+} into the presynaptic terminal. This would suggest that calcium does not modify entry through the calcium channels, but rather some event that occurs after the calcium has already entered the terminal. Experiments which examine the interaction of calcium and release proteins may shed more light on how transmitter release decreases during LTA.

Calcium has been shown by Hong and Lnenicka (1991) to be necessary for the effects of LTA to occur. When reduced calcium was perfused around

the proximal end of the nerve 2 h prior to conditioning, LTA was prevented. This could indicate that Ca^{2+} is required in order for specific proteins to be synthesized, since reduced Ca^{2+} mimicked the effect of CHX, a known inhibitor of protein synthesis. Calcium entry is required both at the proximal and distal ends of the nerve to have the full effect of LTA realized. Calcium must enter at the proximal end during stimulation to produce LTA. The majority of the calcium that enters the nerve terminal is buffered out or exuded from the terminal in a fairly short time span. This means that calcium entering at the nerve terminal should not be able to reach the cell body. Since the cell body has been shown to be the main centre of protein synthesis, calcium may be activating specific genes that regulate LTA.

Work by Lnenicka and Atwood (1989) showed that LTA still occurred if the cell body was stimulated but transmission was blocked by TTX halfway down the axon. This seems to indicate that the cause of LTA is an effect on the cell body which sends some signal to the nerve terminal. If this is the case then it is probably some kind of protein that is modifying functions at the nerve terminal. Further work which examines protein synthesis during LTA would be valuable in trying to determine the causes of this phenomenon. Experiments which examine proteins synthesized prior to stimulation that evokes LTA, with proteins synthesized during and after evoking LTA should show protein(s) that regulate LTA effects.

Conclusions

The central hypothesis of this thesis could not be accepted according to these data. Changes in intrinsic calcium buffers cannot be solely responsible for the decrease in transmitter release during LTA. Since increased calcium influx can mask the effect of BAPTA-AM on EPSP's, the possibility that intrinsic calcium buffers change during LTA cannot be rejected. Also, the finding of seasonal changes in BAPTA-AM effectiveness on transmitter release could influence these data because the experiments were performed over many months.

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